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From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 03 May 2000 (03.05.00)	
International application No. PCT/US99/17116	**Applicant's or agent's file reference** A2996A-WO
International filing date (day/month/year) 28 July 1999 (28.07.99)	**Priority date** (day/month/year) 30 July 1998 (30.07.98)
Applicant YU, Kin, T. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

28 February 2000 (28.02.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Nestor Santesso
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

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PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

15

Applicant's or agent's file reference A2996A-WO	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US99/17116	International filing date *(day/month/year)* 28 JULY 1999	Priority date *(day/month/year)* 30 JULY 1998
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant AVENTIS PHARMACEUTICALS PRODUCTS INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

   [X] This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   - I [X] Basis of the report
   - II [ ] Priority
   - III [ ] Non-establishment of report with regard to novelty, inventive step or industrial applicability
   - IV [ ] Lack of unity of invention
   - V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI [ ] Certain documents cited
   - VII [ ] Certain defects in the international application
   - VIII [X] Certain observations on the international application

CORRECTED VERSION

Date of submission of the demand	Date of completion of this report
28 FEBRUARY 2000	05 SEPTEMBER 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JOHN D. ULM Telephone No. (703) 308-0196

TERRY J. DEY
PARALEGAL SPECIALIST
TECHNOLOGY CENTER 1600

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/17116

I. Basis of the report

1. With regard to the **elements** of the international application:*

☐ the international application as originally filed

☒ the description:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the claims:
pages (See Attached) , as originally filed
pages _____ , as amended (together with any statement) under Article 19
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the drawings:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the sequence listing part of the description:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☒ contained in the international application in printed form.

☒ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages NONE

☒ the claims, Nos. NONE

☒ the drawings, sheets/~~fig~~ NONE

5. ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/17116

V. Reas ned statement under Article 35(2) with regard to novelty, inventive step r industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-50	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-50	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-50	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1 to 50 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest an isolated nucleic acid encoding any of the calcium sensing receptor isoforms disclosed in the instant description.

---------- NEW CITATIONS ----------
NONE

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

The description is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 5 because it fails to adequately enable practice of the claimed invention because: there is no description therein of a method of treating any disorder via gene therapy. The instant description does not identify any disease or disorder which is the consequence of the overexpression or underexpression of a calcium sensing receptor of the instant invention. Further, gene therapy is not a routine practice in the art and the mere disclosure of an isolated nucleic acid encoding a protein does not provide the guidance that would be needed to treat an individual by the administration of either a nucleic acid encoding that protein or a nucleic acid antisense thereto.

Claims 34 to 48 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because practice of the claimed invention is not enabled as required under PCT Rule 5.1(a) for the reasons set forth in the immediately preceding paragraph.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): C07K 14/705, 16/28; C12N 5/10, 15/12 and US Cl.: 435/7.1, 69.1, 252.3, 320.1; 350/350, 388.22; 536/23.5

I. BASIS OF REPORT:

This report has been drawn on the basis of the description,
page(s) 1-42, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the claims,
page(s) 43, 45, 46, as originally filed.
page(s) NONE, as amended under Article 19.
page(s) NONE, filed with the demand.
and additional amendments:
Page 44, filed with the letter of 21 August 2000.

This report has been drawn on the basis of the drawings,
page(s) 1, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the sequence listing part of the description:
page(s) 48-80, as originally filed.
pages(s) NONE, filed with the demand.
and additional amendments:
NONE

retrovirus, adenovirus, adeno-associated virus, herpes virus, and vaccinia virus.

15. A host cell transfected with the vector of claim 10.

16. A host cell transfected with the vector of claim 13.

17. The host cell of claim 15 selected from the group consisting of a bacterial cell, a yeast cell, and a mammalian cell.

18. A method for expressing an isoform of human calcium sensing receptor comprising:

a) culturing the host cell of claim 17 in culture medium under conditions permitting expression of the receptor; and

b) identifying cells expressing the receptor on their surface.

19. An isolated isoform of a human calcium sensing receptor, wherein the isoform comprises about 974 to about 1001 amino acids and has a deletion of about 77 amino acids when compared to the wild-type form of the receptor as depicted in SEQ ID NO:12.

20. The isoform according to claim 19, wherein said deletion is from the extracellular domain of the receptor.

21. The isoform according to claim 20, wherein the deletion is from about amino acids 358-462 of SEQ ID NO:12.

22. The isoform according to claim 20, wherein the deletion is from about amino acids 460-536 of SEQ ID NO:12.

23. The isoform according to claim 20, wherein the deletion is from about amino acids 358-536 of SEQ ID NO:12.

24. The isoform of claim 19, wherein the isoform comprises an amino acid sequence as depicted in SEQ ID NO:8 or SEQ ID NO:10, or allelic variants thereof.

25. A method of screening for agonists or antagonists of a CaSR isoform activity, the method comprising recombinantly producing a CaSR isoform, incubating a test sample with the CaSR isoform, measuring CaSR isoform activity and comparing the activity to that in the absence of the test sample.

26. The method according to claim 25, wherein the CaSR activity is its ability to influence intracellular calcium concentration.

27. The method according to claim 25, wherein the test sample is tested alone, in conjunction with an elevation in extracellular calcium concentration, or in the presence of other agonists or antagonists of CaSR isoform activity.

28. The method according to claim 26, wherein the intracellular calcium concentration is measured with a fluorescent indicator.

29. The method according to claim 28, wherein the indicator is fura-2.

30. A method of treating a patient suffering from a disease or disorder associated with abnormal

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Isoforms of Human Calcium Sensing Receptor

FIELD OF THE INVENTION

The present invention relates to isoforms of a human calcium sensing receptor, and to the genes encoding these isoforms. The invention further relates to methods of screening for agonists or antagonists of the isoforms, particularly with respect to calcium receptor activity, to diagnostic uses of these isoforms and to therapeutic uses of the agonists or antagonists. The invention also relates to gene therapy using the genes encoding the receptor isoforms or molecules capable of down-regulating receptor activity, such as antisense sequences.

BACKGROUND OF THE INVENTION

Calcium is an extracellular messenger (Brown et al. (1995) Cell 83:679-682). Serum calcium levels are regulated by 1,25-dihydroxyvitamin D_3, parathyroid hormone, and calcitonin. A calcium sensing receptor (CaSR) has been identified in bovine parathyroid (WO 94/18959; Brown et al. (1993) Nature 366: 575-580). Cloning of the cDNA encoding this receptor (CaSRa) revealed a G-protein-coupled receptor featuring a large extracellular domain, coupled to a seven membrane spanning domain similar to those found in members of the G protein coupled receptor superfamily. This receptor has been shown to play a key role in Ca^{++} homeostatsis through regulation of parathyroid hormone secretion and renal tubular calcium reabsorption. This receptor recognizes calcium and other polyvalent cations and is coupled by changes in phosphoinositide turnover to the release of calcium from intracellular stores. In addition to its abundant expression in parathyroid gland and kidney, full length CaSRa transcripts have also been found in brain, thyroid, intestine, bone marrow and keratinocytes. The complete cDNA sequence encoding the corresponding human form of CaSRa has recently been reported (Freichel et al. (1996) Endocrinology 137:3842-3848). This 3234 base pair nucleotide sequence (SEQ ID NO: 11) encodes a protein having 1078 amino acids (SEQ ID NO: 12). Various forms of the CaSR, particularly from bone marrow cells, are also disclosed by House et al. ((1997) J. Bone Min. Res. 12:1959-1970) and in US patents 5,688,938 and 5,763,569. The presence of calcium receptors in bone, suggests that they are involved in bone remodeling (Quarles (1997) J. Bone Min. Res. 12, 1971-1974).

An alternatively spliced form of CaSRa, has been identified in human medullary thyroid carcinoma and keratinocytes (see Freichel et al. *supra*). The medullary thyroid carcinoma isoform, designated CaSRb, contains a 307 base pair deletion between nucleotides 186 and 495, corresponding to exon 2. This deletion results in a reading frame shift and premature termination at nucleotide 766. Translation of CaSRb transcript could yield an extracellular portion of the receptor without the 7 transmembrane anchor and cytosolic tail. Therefore, CaSRb may be a secretory protein, and, although its exact function has yet to be determined, by analogy to other known souble receptors it may play a role in

modulating the interaction of native CaSR with its cationic ligands.

A second isoform of CaSRa has also been identified in kerotinocytes (Oda et al. (1997) FASEB J. 11(9): A925; Abstract #395). This form (CaSRc) lacks exon 4, encoding a portion of the extracellular domain of the receptor including a region of acidic amino acids which may mediate calcium binding, and is present in differentiated cells.

However, there is a need in the art to better understand calcium homeostasis. In particular, there is a need in the art to better understand calcium regulation through the CaSR. Isoforms of the wild-type CaSR could be expected to exhibit different pharmacological profiles and signaling properties relative to the wild-type. For example, different isoforms could couple differentially or uniquely to known or unknown signalling pathways, including phosphoinositide turnover, calcium mobilization, protein kinase C activation, cAMP production, and other ion channel activity. Alternatively, variant isoforms may exhibit different cell surface expression, metabolic half-life, or intracellular trafficking, when compared to the wild-type, or behave in a dominant negative or positive fashion, thereby over-riding the functionality of the wild-type receptor expressed in the same tissue.

The present invention addresses the need in the art, as discussed below. Specifically, Applicants present evidence that CaSRb is also found in human kidney. Significantly, Applicants have also identified in human kidney two other alternatively spliced CaSR transcripts (CaSRc and d) with deletions from nucleotides 1378-1608 and 1075 to 1386, respectively.

The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

SUMMARY OF THE INVENTION

As noted above, the present invention concerns identification of isoforms of a human calcium sensing receptor (CaSR). The present invention reveals the presence of mutiple alternatively spliced transcripts of CaSR in human kidney. Two such isoforms identified, CaSRc and d arise from partial deletion of the wild type sequence. The nucleotide deletions in CaSRc and d have no effect on the reading frame. Thus, CaSRc and d will yield receptor proteins with about 90-100 amino acid stretchs deleted from the extracellular domain. These deletions cover a respective extracellular sequence rich in acidic residues (approximately 4%). The location and the charge characteristics of the deleted sequences in CaSRc and d suggest that these two CaSR splice variants may exhibit different cation sensing property from the wild type receptor.

Thus, in a first aspect, the present invention provides isolated nucleic acids encoding an isoform of a human calcium sensing receptor, wherein the nucleic acid comprises about 2922 to about 3003 nucleotides and has a deletion of at least about 231 nucleotides when compared to the wild-type form of the receptor as depicted in SEQ ID NO:11. The term "about" or "approximately" means

within 20%, preferably within 10%, and more preferably within 5% of a given value or range. Preferably, the deletion is in the region encoding the extracellular domain of the receptor.

In a preferred embodiment of the invention, the deletion is from about nucleotides 1075-1386 of SEQ ID NO:11. In another preferred embodiment, the deletion is from about nucleotides 1378-1608 of SEQ ID NO:11. Alternatively, the deletion is from about nucleotides 1075-1608 of SEQ ID NO:11.

In another embodiment, the isolated nucleic acid of the present invention has at least one property selected from:

it can be amplified by polymerase chain reaction (PCR) using an oligonucleotide primer derived from SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11;

it hybridizes under stringent conditions with a nucleic acid having a nucleotide sequence as depicted in SEQ ID NO:7 or SEQ ID NO:9; and

it encodes a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:10, and allelic variants thereof.

Preferably, the nucleic acid the invention can be amplified with at least one oligonucleotide primer selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:6.

In still another preferred embodiment of the invention, the isolated nucleic acid encodes a CaSR isoform comprising an amino acid sequence as depicted in SEQ ID NO:8 (CaSRc) or SEQ ID NO:10 (CaSRd), or allelic variants thereof. Preferably, the isolated nucleic acid comprises a nucleotide sequence as depicted in SEQ ID NO:7 or SEQ ID NO:9, or allelic variants thereof.

As can be readily appreciated by one of ordinary skill in the art, one effective way to prepare a nucleic acid of the invention, particularly a cDNA, is to amplify the nucleic acid from a cDNA library comprising a coding sequence for a CasR isofom using PCR. Various PCR primers, corresponding to any desired segment from SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11 can be used in accordance with the invention. In specific embodiments, *infra*, PCR primers having the sequences depicted in SEQ ID NOS: 1-6 were used to amplify and isolate nucleic acids of the invention. Alternatively, a nucleic acid of the invention can be isolated or identified with an oligonucleotide probe, *e.g.*, of at least 10 bases, which hybridizes under stringent conditions to a nucleotide having the sequence or the complementary sequence depicted in SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11. In a specific aspect, the oligonucleotide can be used in a method for detecting genomic DNA (Southern analysis) or expression of mRNA (Northern analysis) encoding a CaSR isoform in a cell. In either case, the method comprises contacting a sample from the cell with the oligonucleotide which is detectable, *e.g.*, by labeling with a radioisotope or a chromophore or fluorophore, and detecting hybridization of the oligonucleotide with genomic DNA or mRNA in the sample, wherein detection of hybridization of the oligonucleotide with genomic DNA indicates the presence of a gene encoding a CaSR isoform in the genome, and detection of hybridization

with mRNA indicates expression of mRNA encoding a CaSR isoform. It is also possible to use quantitative methods, *e.g.*, to detect the number of *CaSR* genes in the genome, or to detect an increase or decrease in the level of expression of mRNA.

An oligonucleotide of the invention can also be an antisense oligonucleotide, *i.e.*, one that binds to mRNA encoding a CaSR isoform and prevents its translation in the cell. Such an antisense molecule can be encoded by a vector expressed in the cell, or can be a synthetic oligonucleotide, preferably one that includes non-phosphoester bonds so that it is resistant to intracellular nucleases.

In another aspect, the invention provides a vector comprising nucleic acids encoding an isoform of a human calcium sensing receptor. Preferably, the nucleic acid is operatively associated with an expression control sequence permitting expression of the receptor in an expression competent host cell.

The vector may be an RNA molecule, a plasmid DNA molecule, or a viral vector. The viral vector may be a retrovirus, adenovirus, adeno-associated virus, herpes virus, or vaccinia virus.

In still another aspect, the invention is directed to host cells transfected with a vector comprising nucleic acids encoding an isoform of a human calcium sensing receptor. The host cell may be a bacterial cell, a yeast cell, or a mammalian cell. The host cells of the invention can be used to produce a CaSR isoform recombinantly. This method comprises culturing the host cell in culture medium under conditions permitting expression of the isoform. Therefore, another aspect of this invention is a method for expressing an isoform of human calcium sensing receptor comprising:

culturing the host cell in culture medium under conditions permitting expression of the recombinant receptor; and

identifying cells expressing the receptor on their surface.

The invention also provides an isolated isoform of a human calcium sensing receptor, wherein the isoform comprises about 974 to about 1001 amino acids and has a deletion of at least about 77 amino acids when compared to the wild-type form of the receptor as depicted in SEQ ID NO:12. The deletion may be from the extracellular domain of the receptor, such as from about amino acids 358-462, about amino acids 460-536 or about amino acids 358-536 of SEQ ID NO:12. In particular, a CaSR isoform of the invention comprises an amino acid sequence as depicted in SEQ ID NO:8 or SEQ ID NO:10, or allelic variants thereof.

The present invention advantageously provides methods of screening for molecules that modulate the activity of CaSR isoforms, and thus calcium levels. In particular, the invention provides a method of screening for agonists or antagonists of a CaSR isoform activity, the method comprising incubating a test sample with a CaSR isoform, measuring CaSR isoform activity and comparing the activity to that in the absence of the test sample. Any of the screening methods in the art can be used, particularly high throughput screening. In a specific embodiment, the method comprises screening compounds for their

ability to influence intracellular calcium concentration. The compounds may be tested alone, in conjunction with an elevation in extracellular calcium concentration, or in the presence of other agonists or antagonists of CaSR isoform activity. Intracellular calcium can be measured with a fluorescent indicator, such as fura-2. Screening methods of the invention permit the identification of CaSR agonists (calcimimetics) or antagonists (calcilytics).

In yet a further embodiment, the present invention provides pharmaceutical compositions and methods for the treatment of a patient suffering from a disease or disorder associated with abnormal calcium levels, such as in the plasma, by the administration of a therapeutically effective amount of a compound capable of modulating the activity of a CaSR isoform. The compound may be specific for a CaSR isoform. Such diseases include, for example, hyperparathyroidism (primary and secondary) and osteoporosis. Other diseases include Paget's disease, hypercalcemia malignancy, and hypertension. The compound may be a calcimimetic or calcilytic identified using the screening assays of the invention. Alternatively, the disease or disorder is treated using gene therapy.

In one embodiment, the cells of a patient have been transfected with a vector encoding a CaSR isoform under conditions permitting expression of the isoform.

Alternatively, where desired, the invention provides a method of inhibiting CaSR activity in a patient's cell comprising decreasing the level of CaSR in the cell. The level of CaSR protein can be decreased by introducing a CaSR antisense nucleic acid into the cell, which antisense nucleic acid hybridizes under intracellular conditions to a CaSR mRNA. Alternatively, the level of CaSR protein can be decreased by introducing a single chain Fv antibody (scFv) that specifically binds a CaSR isoform, or nucleic acid encoding and intracellular antibody against the isoform, into the cell at a level sufficient to bind to and inactivate the CaSR.

Yet another object of the invention is to provide for high level expression of CaSR isoforms, either by fermentation of transfected or transduced cells to recover purified protein, or *in vivo* in cells for further testing *in vitro* or for regulation of calcium homeostasis *in vivo, e.g.*, for gene therapy.

A particular object of the invention is to provide for screening of small molecule modulators, *e.g.*, agonists and antagonists, of CaSR activity, particularly of specific CaSR isoforms.

These and other objects are addressed by this invention, which is explained in greater detail in the attached drawings and the following Detailed Description and Examples.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. Diagram of the structures of the wild-type calcium sensing receptor (CaSRa), splice variant CaSRd (lacking amino acids encoded by nucleotides 1075-1386), and splice variant CaSRc (lacking amino acids encoded by nucleotides 1378-1608).

DETAILED DESCRIPTION OF THE INVENTION

This invention is based, in part, on the identification of isoforms of a human calcium sensing receptor, termed herein CaSR.

The invention accordingly relates to nucleic acids encoding CaSR isoforms, to the purified protein, to cells which express nucleic acids encoding isoforms of CaSR, in particular splicing variants, and to their use in screening for small molecules or natural products, which agonize or antagonize the activity of the CaSR.

The invention can also be used for the treatment of diseases or disorders associated with an abnormal level of calcium, including gene therapy applications (both coding and antisense molecules can be of use).

In addition, anti-CaSR antibodies can be used in diagnostic and purification applications.

These and other aspects of the invention, particularly isolation of CaSR genes, expression of CaSR protein, generation of anti-CaSR antibodies, screening assays for agonists or antagonists of CaSR activity and delivery of CaSR encoding vectors, in particular for gene therapy applications, are discussed in detail in the following sections. Section headers are provided merely for the reader's convenience, and are not to be deemed limiting in any respect.

Genes Encoding Calcium Sensor Receptor Isoforms

The present invention contemplates isolation of genes encoding isoforms of a human calcium sensing receptor. As used herein, the term "gene" refers to an assembly of nucleotides that encode a polypeptide, and includes cDNA and genomic DNA nucleic acids.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual,* Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); *DNA Cloning: A Practical Approach,* Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "cloning vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA

replication *in vivo*, *i.e.*, capable of replication under its own control. Cloning vectors may be capable of replication in one cell type, and expression in another ("shuttle vector").

A "cassette" refers to a segment of DNA that can be inserted into a vector at specific restriction sites. The segment of DNA encodes a polypeptide of interest, and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation.

A cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. A cell has been "transformed" by exogenous or heterologous DNA when the transfected DNA effects a phenotypic change. The transforming DNA can be integrated (covalently linked) into chromosomal DNA making up the genome of the cell.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester anologs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (*see* Sambrook et al., *supra*). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T_m of 55°, can be used, *e.g.*, 5x SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SSC, 0.5% SDS). Moderate stringency hybridization conditions correspond to a higher T_m, *e.g.*, 40% formamide, with 5x or 6x SCC. High stringency hybridization conditions correspond to the highest T_m, *e.g.*, 50% formamide, 5x or 6x SCC. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater

the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (*see* Sambrook et al., *supra*, 9.50-0.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (*see* Sambrook et al., *supra*, 11.7-11.8). Preferably a minimum length for a hybridizable nucleic acid is at least about 10 nucleotides; preferably at least about 15 nucleotides; and more preferably the length is at least about 20 nucleotides.

In a specific embodiment, the term "standard hybridization conditions" refers to a T_m of 55°C, and utilizes conditions as set forth above. In a preferred embodiment, the T_m is 60°C; in a more preferred embodiment, the T_m is 65°C.

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of at least 18 nucleotides, that is specifically hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule encoding CaSR, or an isoform thereof. Oligonucleotides can be labeled, *e.g.*, with ^{32}P-nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. In one embodiment, a labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid encoding a CaSR, or an isoform thereof. In another embodiment, oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning CaSR isoforms, or to detect the presence of nucleic acids encoding CaSR isoforms. In a further embodiment, an oligonucleotide of the invention can form a triple helix with a CaSR DNA molecule. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer. Accordingly, oligonucleotides can be prepared with non-naturally occurring phosphoester analog bonds, such as thioester bonds, etc.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence. In this case, the nucleic acid is "operatively associated" with an expression control sequence permitting expression of the protein in an expression competent host cell

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced (if the coding sequence contains introns) and translated into the protein encoded by the coding sequence.

As used herein, the term "homologous" in all its grammatical forms and spelling variations refers to the relationship between proteins that possess a "common evolutionary origin," including proteins from superfamilies (*e.g.*, the immunoglobulin superfamily) and homologous proteins from different species (*e.g.*, myosin light chain, etc.) (Reeck et al., 1987, Cell 50:667). Such proteins (and their encoding genes) have sequence homology, as reflected by their high degree of sequence similarity.

Accordingly, the term "sequence similarity" in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that may or may not share a common evolutionary origin (*see* Reeck et al., *supra*). However, in common usage and in the instant application, the term "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and not a common evolutionary origin.

In a specific embodiment, two DNA sequences are "substantially homologous" or "substantially similar" when at least about 50% (preferably at least about 75%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

Similarly, in a particular embodiment, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 30% of the amino acids are identical, or greater than about 60% are similar (functionally identical). Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, *Version 7*, Madison, Wisconsin) pileup program.

The term "corresponding to" is used herein to refer similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured.

A nucleic acid or amino acid sequence alignment may include spaces. Thus, the term "corresponding to" refers to the sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

A gene encoding a CaSR isoform, whether genomic DNA or cDNA, can be isolated from a human cDNA or genomic library. Methods for obtaining genes encoding CaSR isoforms are well known in the art, as described above (*see, e.g.*, Sambrook et al., 1989, *supra*).

Accordingly, any human cell potentially can serve as the nucleic acid source for the molecular cloning of a CaSR isoform gene. The DNA may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"), and preferably is obtained from a cDNA library prepared from tissues with high level expression of the protein (*e.g.*, brain, thyroid and kidney cDNA), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell (See, for example, Sambrook et al., 1989, *supra*; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II). Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will not contain intron sequences. In specific embodiments, isoforms CaSRc and CaSRd were isolated from a human kidney cell library. Whatever the source, the gene should be molecularly cloned into a suitable vector for propagation of the gene.

Once the DNA fragments are generated, identification of the specific DNA fragment containing gene encoding a CaSR isoform may be accomplished in a number of ways. For example, DNA fragments may be screened by nucleic acid hybridization to a labeled probe (Benton and Davis, 1977, Science 196:180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). Those DNA fragments with substantial homology to the probe will hybridize. As noted above, the greater the degree of homology, the more stringent hybridization conditions can be used. In a specific embodiment, Northern hybridization conditions are used to identify mRNA splicing variants of a CaSR gene.

Further selection can be carried out on the basis of the properties of the gene, *e.g.*, if the gene encodes a protein product having the isoelectric, electrophoretic, amino acid composition, or partial amino acid sequence of a CaSR isoform as disclosed herein. Thus, the presence of the gene may be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected which produce a protein that, *e.g.*, has similar or identical electrophoretic migration, isoelectric focusing or non-equilibrium pH gel electrophoresis behavior, proteolytic digestion maps, or antigenic properties as known for CaSR. In a specific embodiment, the isoform is recognized by a polyclonal antibody that does not recognize wild-type CaSR.

The present invention relates to genes (*e.g.*, cDNAs) encoding allelic variants, splicing variants, analogs, and derivatives of CaSR isoforms of the invention that have the same or homologous functional activity as the isoforms. The production and use of derivatives and analogs related to CaSR isoforms are

within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, *i.e.*, capable of exhibiting one or more functional activities associated with an isoform of the invention. In particular, such an analog can bind calcium. Alternatively, an allelic variant can comprise a mutation that results an inability to bind calcium.

Derivatives can be made by altering encoding nucleic acid sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Preferably, derivatives are made that have enhanced or increased functional activity relative to native CaSR isoforms.

Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a gene encoding a CaSR isoform, including an amino acid sequence that contains a single amino acid variant, may be used in the practice of the present invention. These include but are not limited to allelic genes, homologous genes from other species, and nucleotide sequences comprising all or portions of CaSR isoform genes which are altered by the substitution of different codons that encode the same amino acid residue within the sequence, thus producing a silent change. Likewise, the derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a CaSR isoform including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a conservative amino acid substitution. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. Amino acids containing aromatic ring structures are phenylalanine, tryptophan, and tyrosine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations will not be expected to affect apparent molecular weight as determined by polyacrylamide gel electrophoresis, or isoelectric point.

Particularly preferred substitutions are:

- Lys for Arg and vice versa such that a positive charge may be maintained;
- Glu for Asp and vice versa such that a negative charge may be maintained;
- Ser for Thr such that a free -OH can be maintained; and
- Gln for Asn such that a free $CONH_2$ can be maintained.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (*i.e.*, His can act as an acid or base

and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces b-turns in the protein's structure.

The genes encoding CaSR isoforms, derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned gene sequence can be modified by any of numerous strategies known in the art (Sambrook et al., 1989, *supra*). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding an CaSR isoform, derivative or analog, care should be taken to ensure that the modified gene remains within the same translational reading frame as the CaSR gene (SEQ ID NO:11), uninterrupted by translational stop signals, in the gene region where the desired activity is encoded.

Additionally, the CaSR isoform-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Preferably, such mutations enhance the functional activity of the mutated gene product. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem. 253:6551; Zoller and Smith, 1984, DNA 3:479-488; Oliphant et al., 1986, Gene 44:177; Hutchinson et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:710), use of TAB® linkers (Pharmacia), etc. PCR techniques are preferred for site directed mutagenesis (see Higuchi, 1989, "Using PCR to Engineer DNA", in *PCR Technology: Principles and Applications for DNA Amplification*, H. Erlich, ed., Stockton Press, Chapter 6, pp. 61-70).

The identified and isolated gene can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Examples of vectors include, but are not limited to, *E. coli*, bacteriophages such as lambda derivatives, or plasmids such as pBR322 derivatives or pUC plasmid derivatives, *e.g.*, pGEX vectors, pmal-c, pFLAG, etc. The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated. Preferably, the cloned gene is contained on a shuttle vector plasmid, which provides for expansion in a cloning cell, *e.g.*, *E. coli*, and

facile purification for subsequent insertion into an appropriate expression cell line, if such is desired. For example, a shuttle vector, which is a vector that can replicate in more than one type of organism, can be prepared for replication in both *E. coli* and *Saccharomyces cerevisiae* by linking sequences from an *E. coli* plasmid with sequences form the yeast 2m plasmid.

Expression of CaSR Isoforms

The nucleotide sequence coding for CaSR isoforms, or derivatives or analogs thereof, including a chimeric protein, can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. Such elements are termed herein a "promoter." Thus, the nucleic acid encoding a CaSR isoform of the invention is operationally associated with a promoter in an expression vector of the invention. Both cDNA and genomic sequences can be cloned and expressed under control of such regulatory sequences. An expression vector also preferably includes a replication origin.

The necessary transcriptional and translational signals can be provided on a recombinant expression vector, or they may be supplied by the native gene encoding a CaSR, a CaSR isoform and/or its flanking regions.

Potential host-vector systems include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

A recombinant CaSR isoform of the invention, derivative, or analog thereof, may be expressed chromosomally, after integration of the coding sequence by recombination. In this regard, any of a number of amplification systems may be used to achieve high levels of stable gene expression (*See* Sambrook et al., 1989, *supra*).

The cell into which the recombinant vector comprising the nucleic acid encoding a CaSR isoform is cultured in an appropriate cell culture medium under conditions that provide for expression of protein by the cell.

Any of the methods previously described for the insertion of DNA fragments into a cloning vector may be used to construct expression vectors containing a gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombination (genetic recombination).

Expression of a gene may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be

used to control gene expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the b-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the *tac* promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and the animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58), alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94), myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Expression vectors containing a nucleic acid encoding a CaSR isoform of the invention can be identified by five general approaches: (a) PCR amplification of the desired plasmid DNA or specific mRNA, (b) nucleic acid hybridization, (c) presence or absence of selection marker gene functions, (d) analyses with appropriate restriction endonucleases, and (e) expression of inserted sequences. In the first approach, the nucleic acids can be amplified by PCR to provide for detection of the amplified product. In the second approach, the presence of a foreign gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted marker gene. In the third approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "selection marker" gene functions (*e.g.*, b-galactosidase activity,

thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. In another example, if the nucleic acid encoding a CaSR isoform is inserted within the "selection marker" gene sequence of the vector, recombinants containing the nucleic acid insert can be identified by the absence of the gene function. In the fourth approach, recombinant expression vectors are identified by digestion with appropriate restriction enzymes. In the fifth approach, recombinant expression vectors can be identified by assaying for the activity, biochemical, or immunological characteristics of the gene product expressed by the recombinant, provided that the expressed protein assumes a functionally active conformation.

A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, *e.g., E. coli* plasmids col El, pCR1, pBR322, pMal-C2, pET, pGEX (Smith *et al.*, 1988, Gene 67:31-40), pMB9 and their derivatives, plasmids such as RP4; phage DNAS, e.g., the numerous derivatives of phage l, e.g., NM989, and other phage DNA, *e.g.*, M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2m plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

For example, in a baculovirus expression systems, both non-fusion transfer vectors, such as but not limited to pVL941 (*Bam*H1 cloning site; Summers), pVL1393 (*Bam*H1, *Sma*I, *Xba*I, *Eco*R1, *Not*I, *Xma*III, *Bgl*II, and *Pst*I cloning site; Invitrogen), pVL1392 (*Bgl*II, *Pst*I, *Not*I, *Xma*III, *Eco*RI, *Xba*I, *Sma*I, and *Bam*H1 cloning site; Summers and Invitrogen), and pBlue*Bac*III (*Bam*H1, *Bgl*II, *Pst*I, *Nco*I, and *Hind*III cloning site, with blue/white recombinant screening possible; Invitrogen), and fusion transfer vectors, such as but not limited to pAc700 (*Bam*H1 and *Kpn*I cloning site, in which the *Bam*H1 recognition site begins with the initiation codon; Summers), pAc701 and pAc702 (same as pAc700, with different reading frames), pAc360 (*Bam*H1 cloning site 36 base pairs downstream of a polyhedrin initiation codon; Invitrogen(195)), and pBlueBacHisA, B, C (three different reading frames, with *Bam*H1, *Bgl*II, *Pst*I, *Nco*I, and *Hind*III cloning site, an N-terminal peptide for ProBond purification, and blue/white recombinant screening of plaques; Invitrogen (220)) can be used.

Mammalian expression vectors contemplated for use in the invention include vectors with inducible promoters, such as the dihydrofolate reductase (DHFR) promoter, *e.g.*, any expression vector with a *DHFR* expression vector, or a *DHFR*/methotrexate co-amplification vector, such as pED (*Pst*I, *Sal*I, *Sba*I, *Sma*I, and *Eco*RI cloning site, with the vector expressing both the cloned gene and *DHFR*; *see* Kaufman, *Current Protocols in Molecular Biology*, 16.12 (1991). Alternatively, a glutamine synthetase/methionine sulfoximine co-amplification vector, such as pEE14 (*Hind*III, *Xba*I, *Sma*I, *Sba*I,

*Eco*RI, and *Bcl*I cloning site, in which the vector expresses glutamine synthase and the cloned gene; Celltech). In another embodiment, a vector that directs episomal expression under control of Epstein Barr Virus (EBV) can be used, such as pREP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive Rous Sarcoma Virus Long Terminal Repeat (RSV-LTR) promoter, hygromycin selectable marker; Invitrogen), pCEP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive human cytomegalovirus (hCMV) immediate early gene, hygromycin selectable marker; Invitrogen), pMEP4 (*Kpn*I, *Pvu*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, *Bam*H1 cloning site, inducible methallothionein IIa gene promoter, hygromycin selectable marker: Invitrogen), pREP8 (*Bam*H1, *Xho*I, *Not*I, *Hind*III, *Nhe*I, and *Kpn*I cloning site, RSV-LTR promoter, histidinol selectable marker; Invitrogen), pREP9 (*Kpn*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, and BamHI cloning site, RSV-LTR promoter, G418 selectable marker; Invitrogen), and pEBVHis (RSV-LTR promoter, hygromycin selectable marker, N-terminal peptide purifiable via ProBond resin and cleaved by enterokinase; Invitrogen). Selectable mammalian expression vectors for use in the invention include pRc/CMV (*Hind*III, *Bst*XI, *Not*I, *Sba*I, and *Apa*I cloning site, G418 selection; Invitrogen), pRc/RSV (*Hind*III, *Spe*I, *Bst*XI, *Not*I, *Xba*I cloning site, G418 selection; Invitrogen), and others. Vaccinia virus mammalian expression vectors (*see*, Kaufman, 1991, *supra*) for use according to the invention include but are not limited to pSC11 (*Sma*I cloning site, TK- and b-gal selection), pMJ601 (*Sal*I, *Sma*I, *Afl*I, *Nar*I, *Bsp*MII, *Bam*HI, *Apa*I, *Nhe*I, *Sac*II, *Kpn*I, and *Hind*III cloning site; TK- and b-gal selection), and pTKgptF1S (*Eco*RI, *Pst*I, *Sal*I, *Acc*I, *Hind*II, *Sba*I, *Bam*HI, and Hpa cloning site, TK or XPRT selection).

Yeast expression systems can also be used according to the invention to express isoforms of a CaSR. For example, the non-fusion pYES2 vector (*Xba*I, *Sph*I, *Sho*I, *Not*I, *Gst*XI, *Eco*RI, *Bst*XI, *Bam*H1, *Sac*I, *Kpn*1, and *Hind*III cloning sit; Invitrogen) or the fusion pYESHisA, B, C (*Xba*I, *Sph*I, *Sho*I, *Not*I, *Bst*XI, *Eco*RI, *Bam*H1, *Sac*I, *Kpn*I, and *Hind*III cloning site, N-terminal peptide purified with ProBond resin and cleaved with enterokinase; Invitrogen), to mention just two, can be employed according to the invention.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Different host cells have characteristic and specific mechanisms for the translational and post-translational processing and

modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. Expression in yeast can produce a biologically active product. Expression in eukaryotic cells can increase the likelihood of "native" folding. Moreover, expression in mammalian cells can provide a tool for reconstituting, or constituting, CaSR activity. Furthermore, different vector/host expression systems may affect processing reactions, such as proteolytic cleavages, to a different extent.

Vectors are introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter (see, *e.g.*, Wu et al., 1992, J. Biol. Chem. 267:963-967; Wu and Wu, 1988, J. Biol. Chem. 263:14621-14624; Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990).

Soluble forms of the protein can be obtained by collecting culture fluid, or solubilizing inclusion bodies, *e.g.*, by treatment with detergent, and if desired sonication or other mechanical processes, as described above. The solubilized or soluble protein can be isolated using various techniques, such as polyacrylamide gel electrophoresis (PAGE), isoelectric focusing, 2-dimensional gel electrophoresis, chromatography (*e.g.*, ion exchange, affinity, immunoaffinity, and sizing column chromatography), centrifugation, differential solubility, immunoprecipitation, or by any other standard technique for the purification of proteins.

Antibodies to CaSR Isoforms

The invention provides an antibody which specifically binds a CaSR isoform. Such antibodies can be used diagnostically, to detect the presence and optionally the quantity of the isoform in cells. Antibodies of the invention, particularly single chain Fv antibodies (scFv) can also be used therapeutically, to suppress CaSR activity (see below). In a specific embodiment, the antibody recognizes an epitope which is not present in the wild type receptor, CaSRa. In another specific embodiment, exemplified *infra*, the antibody is polyclonal. Monoclonal antibodies, and antibody fragments (in addition to scFv antibodies) are also contemplated by this invention. Using the antibody of the invention, one can specifically detect expression of a CaSR isoform in a cell by contacting a sample from the cell with the antibody under conditions permitting binding of the antibody to the protein in the sample, and detecting binding of the antibody to a protein in the sample, wherein detection of binding of the antibody to the protein indicates expression of a CaSR isoform in the cell. Using quantitative immunoassay or Western blotting methods, it is possible to quantitate the amount of CaSR, and particularly to detect increases or decreases in the amount of CaSR relative to the cell at an earlier time, or to normal cells.

According to the invention, a human CaSR isoform produced recombinantly or by chemical synthesis, and fragments or other derivatives or analogs thereof, including fusion proteins, may be used as

an antigen or immunogen to generate antibodies that recognize the polypeptide. A molecule is "antigenic" when it is capable of specifically interacting with an antigen recognition molecule of the immune system, such as an immunoglobulin (antibody) or T cell antigen receptor. An antigenic polypeptide contains at least about 5, and preferably at least about 10, amino acids. An antigenic portion of a molecule can be that portion that is immunodominant for antibody or T cell receptor recognition, or it can be a portion used to generate an antibody to the molecule by conjugating the antigenic portion to a carrier molecule for immunization. A molecule that is antigenic need not be itself immunogenic, *i.e.*, capable of eliciting an immune response without a carrier. Preferably, the antigenic polypeptide comprises an epitope and/or a sequence not present in the wild-type CaSRa, and elicits antibodies which bind to a CaSR isoform.

Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. The antibodies of the invention may be cross reactive, *e.g.*, they may recognize CaSR isoforms from different species. Polyclonal antibodies have greater likelihood of cross reactivity. Alternatively, an antibody of the invention may be specific for a single isoform of CaSR. In a preferred embodiment, the antibodies are capable of specifically recognizing an isoform of CaSR, and are not capable of recognizing the wild-type CaSR.

Various procedures known in the art may be used for the production of polyclonal antibodies. For the production of antibody, various host animals can be immunized by injection with the CaSR isoform, or a derivative (*e.g.*, fragment or fusion protein) thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc. In one embodiment, a polypeptide or fragment thereof can be conjugated to an immunogenic carrier, *e.g.*, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*.

For preparation of monoclonal antibodies against the isoform, or fragment, analog, or derivative thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler and Milstein [*Nature* 256:495-497 (1975)], as well as the trioma technique, the human B-cell hybridoma technique [Kozbor et al., *Immunology Today* 4:72 1983); Cote et al., *Proc. Natl. Acad. Sci. U.S.A.* 80:2026-2030 (1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole et al., in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)]. In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals [International Patent Publication No. WO 89/12690, published 28 December 1989]. In fact, according to the invention, techniques developed for the production of "chimeric antibodies" [Morrison et

al., *J. Bacteriol.* 159:870 (1984); Neuberger et al., *Nature* 312:604-608 (1984); Takeda et al., *Nature* 314:452-454 (1985)] by splicing the genes from a mouse antibody molecule specific for a CaSR isoform together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. Such human or humanized chimeric antibodies are preferred for use in therapy of human diseases or disorders (described *infra*), since the human or humanized antibodies are much less likely than xenogenic antibodies to induce an immune response, in particular an allergic response, themselves.

According to the invention, techniques described for the production of single chain Fv (scFv) antibodies [U.S. Patent Nos. 5,476,786 and 5,132,405 to Huston; U.S. Patent 4,946,778] can be adapted to produce CaSR isoform-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries [Huse et al., *Science* 246:1275-1281 (1989)] to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for a CaSR isoform.

Antibody fragments which contain the idiotype of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. For example, to select antibodies which recognize a specific epitope of a CaSR isoform, one may assay generated hybridomas for a product which binds to an isoform containing such epitope. For selection of an antibody specific to an isoform from a particular species of animal, one can select on the basis of positive binding with a CaSR isoform expressed by or isolated from cells of that species of animal, but which does not bind the wild-type CaSR.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the CaSR isoform, *e.g.*, for Western blotting, imaging theisoform *in situ*, measuring levels

thereof in appropriate physiological samples, etc. using any of the detection techniques mentioned above or known in the art.

In a specific embodiment, antibodies that agonize or antagonize the activity of CaSR, and, in particular, are specific for a CaSR isoform, can be generated. Such antibodies can be tested using the assays described *infra* for identifying ligands. In particular, such antibodies can be scFv antibodies expressed intracellularly.

Screening Assays

Identification and isolation of a gene encoding isoforms of a CaSR of the invention provides for expression of these isoforms in quantities greater than can be isolated from natural sources, or in indicator cells that are specially engineered to indicate CaSR activity after transfection or transformation of the cells. Accordingly, in addition to rational design of agonists and antagonists based on the structures of CaSR isoforms, the present invention contemplates an alternative method for identifying specific ligands of CaSR isoforms using various screening assays known in the art.

Any screening technique known in the art can be used to screen for CaSR agonists or antagonists. The present invention contemplates screens for small molecule ligands or ligand analogs and mimics, as well as screens for natural ligands that bind to and agonize (calcimimetics) or antagonize (calcilytics) CaSR activity *in vivo*. For example, natural products libraries can be screened using assays of the invention for molecules that agonize or antagonize CaSR activity. The present invention provides both the means and methodology for identifying compounds capable of modulating CaSR activity, including the specific modulation of CaSR isoforms. Screening assays for calcimimetics and calcilytics are discussed in WO 94/18959 and US Pat. No. 5,763,569, the entire contents of which are incorporated herein by reference.

In a preferred screening assay compounds are assayed for their ability to influence intracellular calcium concentration. The compounds may be tested alone, in conjunction with an elevation in extracellular calcium concentration, or in the presence of other agonists or antagonists of CaSR isoform activity. Agonists include neomycin, di- and tri-valent cations (gadolinium, calcium, magnesium, strontium, barium, lanthanum), polyamines and other known calcimimetics. Intracellular calcium is measured with the fluorescent indicator, fura-2 (from Molecular Probes). For example, HEK-293 cells transfected with a nucleic acid encoding a CaSR isoform is loaded in buffer containing 0.5uM fura-2, 20mM HEPES, pH 7.35, 0.1% BSA, 0.5mM $CaCl_2$, 0.5mM $MgCl_2$, 6.7mM KCl, 3mM glucose and 142mM NaCl for 45 min at 37°C. The cells are washed and resuspended to about 2 x 10^6 cells/ml in the loading buffer without fura-2. For intracellular calcium measurement, cells were placed in a quartz cuvette equilibrated at 37°C. Fluorescence is detected using excitation monochrometers centered at 340 and 380 nm and emission light collected at 505 nm.

Knowledge of the primary sequence of CaSR isoforms, and the similarity of their sequences with proteins of known function, can provide an initial clue as the inhibitors or antagonists of the protein. Identification and screening of antagonists is further facilitated by determining structural features of the protein, *e.g.*, using X-ray crystallography, neutron diffraction, nuclear magnetic resonance spectrometry, and other techniques for structure determination. These techniques provide for the rational design or identification of agonists and antagonists.

Another approach uses recombinant bacteriophage to produce large libraries. Using the "phage method" [Scott and Smith, 1990, *Science* 249:386-390 (1990); Cwirla, et al., *Proc. Natl. Acad. Sci.*, 87:6378-6382 (1990); Devlin et al., *Science*, 249:404-406 (1990)], very large libraries can be constructed (10^6-10^8 chemical entities). A second approach uses primarily chemical methods, of which the Geysen method [Geysen et al., *Molecular Immunology* 23:709-715 (1986); Geysen et al. *J. Immunologic Method* 102:259-274 (1987)] and the method of Fodor et al. [*Science* 251:767-773 (1991)] are examples. Furka et al. [*14th International Congress of Biochemistry, Volume 5*, Abstract FR:013 (1988); Furka, *Int. J. Peptide Protein Res.* 37:487-493 (1991)], Houghton [U.S. Patent No. 4,631,211, issued December 1986] and Rutter et al. [U.S. Patent No. 5,010,175, issued April 23, 1991] describe methods to produce a mixture of peptides that can be tested as agonists or antagonists.

In another aspect, synthetic libraries [Needels et al., *Proc. Natl. Acad. Sci. USA* 90:10700-4 (1993); Ohlmeyer et al., *Proc. Natl. Acad. Sci. USA* 90:10922-10926 (1993); Lam et al., International Patent Publication No. WO 92/00252; Kocis et al., International Patent Publication No. WO 9428028, each of which is incorporated herein by reference in its entirety], and the like can be used to screen for CaSR ligands according to the present invention.

The screening can be performed with recombinant cells that express a CaSR isoform, or alternatively, using purified protein, *e.g.*, produced recombinantly, as described above. For example, the ability of a labeled, soluble CaSR isoform that includes the extracellular calcium binding portion of the molecule, can be used to screen libraries, as described in the foregoing references.

In one embodiment, a CaSR isoform may be directly labeled. In another embodiment, a labeled secondary reagent may be used to detect binding of an isoform to a molecule of interest, *e.g.*, a molecule attached to a solid phase support. Binding may be detected by *in situ* formation of a chromophore by an enzyme label. Suitable enzymes include, but are not limited to, alkaline phosphatase and horseradish peroxidase. In a further embodiment, a two color assay, using two chromogenic substrates with two enzyme labels on different acceptor molecules of interest, may be used. Cross-reactive and singly-reactive ligands may be identified with a two-color assay.

Other labels for use in the invention include colored latex beads, magnetic beads, fluorescent labels (e.g., fluorescene isothiocyanate (FITC), phycoerythrin (PE), Texas red (TR), rhodamine, free or chelated lanthanide series salts, especially Eu^{3+}, to name a few fluorophores), chemiluminescent

molecules, radio-isotopes, or magnetic resonance imaging labels. Two color assays may be performed with two or more colored latex beads, or fluorophores that emit at different wavelengths. Labeled may be detected visually or by mechanical/optical means. Mechanical/optical means include fluorescence activated sorting, *i.e.*, analogous to FACS, and micromanipulator removal means.

As exemplified herein, the level of the CaSR isoform can be evaluated by metabolic labeling of the proteins. As the metabolic labeling occurs during *in vitro* incubation of the tissue biopsy in the presence of culture medium supplemented with [^{35}S]-methionine, the level of each of the markers detected may be affected by the *in vitro* conditions. In addition to metabolic (or biosynthetic) labeling with [^{35}S]-methionine, the invention further contemplates labeling with [^{14}C]-amino acids and [^{3}H]-amino acids (with the tritium substituted at non-labile positions). Thus, a sample or library of compounds can be directly analyzed after labeling of the proteins therein, *e.g.*, by colorimetric staining using silver, gold, coomassie blue, or amido-schwartz, to mention a few techniques; isotopic labeling, *e.g.*, with [^{32}P]-orthophosphate, [^{125}I], [^{131}I]; fluorescent or chemiluminescent tags; and immunological detection with labeled antibody or specific binding partner of a marker.

Pharmaceutical Compositions and Therapy

Diseases or disorders associated with calcium homeostasis, and therefore, the CaSR, are known in the art. Such diseases are related to the functional responses of cells to calcium, such as parathyroid hormone secretion from parathyroid cells, calcitonin secretion by C-cells and bone resorption by osteoclasts. An example is hyperparathyroidism, which results in elevated levels of parathyroid hormone in the plasma. Therefore, a method of decreasing plasma parathyroid hormone levels is a way of treating hyperparathyroidism. Alternatively, increased levels of plasma calcitonin are associated with inhibition of bone resorption. Inhibition of bone resorption offers a way to treat osteoporosis, for example. The present invention provides both the means and methodology for identifying compounds capable of modulating CaSR activity, including the specific modulation of CaSR isoforms, and of using these compounds for the treatment of diseases or disorders associated with abnormal calcium levels.

Therefore, the present invention provides pharmaceutical compositions and methods for the treatment of a patient suffering from a disease or disorder associated with abnormal calcium levels, such as in the plasma, by the administration of a therapeutically effective amount of a compound capable of modulating the activity of a CaSR isoform. The term "patient" includes both human and other mammals.

"Pharmaceutical composition" refers to a composition comprising the compound and at least one component selected from the group comprising pharmaceutically acceptable carriers, diluents, adjuvants, excipients, or vehicles, such as preserving agents, fillers, disintegrating agents, wetting agents, emulsifying agents, suspending agents, sweetening agents, flavoring agents, perfuming agents,

antibacterial agents, antifungal agents, lubricating agents and dispensing agents, depending on the nature of the mode of administration and dosage forms. Examples of suspending agents include ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, or mixtures of these substances. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, for example sugars, sodium chloride and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monosterate and gelatin. Examples of suitable carriers, diluents, solvents or vehicles include water, ethanol, polyols, suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Examples of excipients include lactose, milk sugar, sodium citrate, calcium carbonate, dicalcium phosphate phosphate. Examples of disintegrating agents include starch, alginic acids and certain complex silicates. Examples of lubricants include magnesium stearate, sodium lauryl sulphate, talc, as well as high molecular weight polyethylene glycols.

"Pharmaceutically acceptable" means it is, within the scope of sound medical judgement, suitable for use in contact with the cells of humans and lower animals without undue toxicity, irritation, allergic response and the like, and are commensurate with a reasonable benefit/risk ratio.

"Pharmaceuticallly acceptable dosage forms" refers to dosage forms of the compound of the invention, and includes, for example, tablets, dragees, powders, elixirs, syrups, liquid preparations, including suspensions, sprays, inhalants tablets, lozenges, emulsions, solutions, granules, capsules and suppositories, as well as liquid preparations for injections, including liposome preparations. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, latest edition.

"Pharmaceutically acceptable salts" refers to the relatively non-toxic, inorganic and organic acid addition salts, and base addition salts, of compounds of the present invention. These salts can be prepared *in situ* during the final isolation and purification of the compounds. In particular, acid addition salts can be prepared by separately reacting the purified compound in its free base form with a suitable organic or inorganic acid and isolating the salt thus formed. Exemplary acid addition salts include the hydrobromide, hydrochloride, sulfate, bisulfate, phosphate, nitrate, acetate, oxalate, valerate, oleate, palmitate, stearate, laurate, borate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphthylate, mesylate, glucoheptonate, lactiobionate, sulphamates, malonates, salicylates, propionates, methylene-bis-b-hydroxynaphthoates, gentisates, isethionates, di-p-toluoyltartrates, methane-sulphonates, ethanesulphonates, benzenesulphonates, p-toluenesulphonates, cyclohexylsulphamates and quinateslaurylsulphonate salts, and the like. (See, for example S. M. Berge, et al., "Pharmaceutical Salts," J. Pharm. Sci., 66: p.1-19 (1977) which is incorporated herein by reference.) Base addition salts can also be prepared by separately reacting the purified compound in its acid form with a suitable organic or inorganic base and isolating the salt thus

formed. Base addition salts include pharmaceutically acceptable metal and amine salts. Suitable metal salts include the sodium, potassium, calcium, barium, zinc, magnesium, and aluminum salts. The sodium and potassium salts are preferred. Suitable inorganic base addition salts are prepared from metal bases which include sodium hydride, sodium hydroxide, potassium hydroxide, calcium hydroxide, aluminium hydroxide, lithium hydroxide, magnesium hydroxide, zinc hydroxide. Suitable amine base addition salts are prepared from amines which have sufficient basicity to form a stable salt, and preferably include those amines which are frequently used in medicinal chemistry because of their low toxicity and acceptability for medical use. ammonia, ethylenediamine, N-methyl-glucamine, lysine, arginine, ornithine, choline, N,N'-dibenzylethylenediamine, chloroprocaine, diethanolamine, procaine, N-benzylphenethylamine, diethylamine, piperazine, tris(hydroxymethyl)-aminomethane, tetramethylammonium hydroxide, triethylamine, dibenzylamine, ephenamine, dehydroabietylamine, N-ethylpiperidine, benzylamine, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, ethylamine, basic amino acids, e.g., lysine and arginine, and dicyclohexylamine, and the like.

"Solid dosage form" means the dosage form of the compound of the invention is solid form, for example capsules, tablets, pills, powders, dragees or granules. In such solid dosage forms, the compound of the invention is admixed with at least one inert customary excipient (or carrier) such as sodium citrate or dicalcium phosphate or (a) fillers or extenders, as for example, starches, lactose, sucrose, glucose, mannitol and silicic acid, (b) binders, as for example, carboxymethylcellulose, alignates, gelatin, polyvinylpyrrolidone, sucrose and acacia, (c) humectants, as for example, glycerol, (d) disintegrating agents, as for example, agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain complex silicates and sodium carbonate, (e) solution retarders, as for example paraffin, (f) absorption accelerators, as for example, quaternary ammonium compounds, (g) wetting agents, as for example, cetyl alcohol and glycerol monostearate, (h) adsorbents, as for example, kaolin and bentonite, (i) lubricants, as for example, talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, (j) opacifying agents, (k) buffering agents , and agents which release the compound(s) of the invention in a certain part of the intestinal tract in a delayed manner.

The choice of vehicle and the content of active substance in the vehicle are generally determined in accordance with the solubility and chemical properties of the active compound, the particular mode of administration and the provisions to be observed in pharmaceutical practice. For example, excipients such as lactose, sodium citrate, calcium carbonate, dicalcium phosphate and disintegrating agents such as starch, alginic acids and certain complex silicates combined with lubricants such as magnesium stearate, sodium lauryl sulphate and talc may be used for preparing tablets. To prepare a capsule, it is advantageous to use lactose and high molecular weight polyethylene glycols. When aqueous suspensions are used they can contain emulsifying agents or agents which facilitate suspension. Diluents such as sucrose, ethanol, polyethylene glycol, propylene glycol, glycerol and chloroform or mixtures thereof may also be used.

The oily phase of the emulsions of this invention may be constituted from known ingredients in a known manner. While the phase may comprise merely an emulsifier (otherwise known as an emulgent), it desirably comprises a mixture of at least one emulsifier with a fat or an oil or with both a fat and an oil. Preferably, a hydrophilic emulsifier is included together with a lipophilic emulsifier which acts as a stabilizer. It is also preferred to include both an oil and a fat. Together, the emulsifier(s) with or without stabilizer(s) make up the emulsifying wax, and the way together with the oil and fat make up the emulsifying ointment base which forms the oily dispersed phase of the cream formulations. Emulgents and emulsion stabilizers suitable for use in the formulation of the present invention include Tween® 60, Span® 80, cetostearyl alcohol, benzyl alcohol, myristyl alcohol, glyceryl mono-stearate and sodium lauryl sulfate.

If desired, the aqueous phase of the cream base may include, for example, a least 30% w/w of a polyhydric alcohol, i.e. an alcohol having two or more hydroxyl groups such as propylene glycol, butane 1,3-diol, mannitol, sorbitol, glycerol and polyethylene glycol (including PEG 400) and mixtures thereof. The topical formulations may desirably include a compound which enhances absorption or penetration of the active ingredient through the skin or other affected areas. Examples of such dermal penetration enhancers include dimethyl sulphoxide and related analogs.

The choice of suitable oils or fats for the formulation is based on achieving the desired cosmetic properties. Thus the cream should preferably be a non-greasy, non-staining and washable product with suitable consistency to avoid leakage from tubes or other containers. Straight or branched chain, mono- or dibasic alkyl esters such as di-isopropyl myristate, decyl oleate, isopropyl palmitate, butyl stearate, 2-ethylhexyl palmitate or a blend of branched chain esters known as Crodamol CAP may be used, the last three being preferred esters. These may be used alone or in combination depending on the properties required. Alternatively, high melting point lipids such as white soft paraffin and/or liquid paraffin or other mineral oils can be used.

Solid compositions of may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols, and the like.

The pharmaceutical compositions can be administered in a suitable formulation to humans and animals by topical or systemic administration, including oral, inhalational, rectal, nasal, buccal, sublingual, vaginal, parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural), intracisternal and intraperitoneal. It will be appreciated that the preferred route may vary with for example the condition of the recipient.

The formulations can be prepared in unit dosage form by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

"Formulations suitable for oral administration" may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tables may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compounds moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein.

Solid compositions for rectal administration include suppositories formulated in accordance with known methods and containing at least one compound of the invention.

If desired, and for more effective distribution, the compounds can be microencapsulated in, or attached to, a slow release or targeted delivery systems such as a biocompatible, biodegradable polymer matrices (e.g. poly(d,l-lactide co-glycolide)), liposomes, and microspheres and subcutaneously or intramuscularly injected by a technique called subcutaneous or intramuscular depot to provide continuous slow release of the compound(s) for a period of 2 weeks or longer. The compounds may be sterilized, for example, by filtration through a bacteria retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved in sterile water, or some other sterile injectable medium immediately before use.

Actual dosage levels of active ingredient in the compositions of the invention may be varied so as to obtain an amount of active ingredient that is effective to obtain a desired therapeutic response for a particular composition and method of administration. The selected dosage level therefore depends upon the desired therapeutic effect, on the route of administration, on the desired duration of treatment and other factors.

Total daily dose of the compounds of this invention administered to a host in single or divided doses may be in amounts, for example, of from about 0.001 to about 100 mg/kg body weight daily and preferably 0.01 to 10 mg/kg/day. Dosage unit compositions may contain such amounts of such submultiples thereof as may be used to make up the daily dose. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the body weight, general health, sex, diet, time and route of administration, rates of absorption and excretion, combination with other drugs and the severity of the particular disease being treated.

The amount of each component administered is determined by the attending clinicians taking into consideration the etiology and severity of the disease, the patient's condition and age, the potency of each component and other factors.

The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials with elastomeric stoppers, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Gene Therapy and Transgenic Vectors

The present invention also relates to gene therapy of diseases or disorders associated with abnormal levels of calcium. As discussed above, a "vector" is any means for the transfer of a nucleic acid according to the invention into a host cell. Preferred vectors are viral vectors, such as retroviruses, herpes viruses, adenoviruses, and adeno-associated viruses. Thus, a gene encoding a CaSR, a CaSR isoform, a polypeptide domain fragment thereof, or a nucleic acid encoding a CaSR antisense sequence is introduced *in vivo*, *ex vivo*, or *in vitro* using a viral vector or through direct introduction of DNA. Expression in targeted tissues can be effected by targeting the transgenic vector to specific cells, such as with a viral vector or a receptor ligand, or by using a tissue-specific promoter, or both.

Expression vectors of the invention can be used, as pointed out above, both to transfect cells for screening or biological testing of modulators of CaSR activity, or for delivery of a CaSR nucleic acid, as described above, or CaSR antisense gene *in vivo* or *ex vivo* for gene therapy, *e.g.*, to increase or decrease the level of CaSR activity. A vector that expresses an anti-CaSR scFv can also be introduced using the techniques discussed below.

Viral vectors commonly used for *in vivo* or *ex vivo* targeting and therapy procedures are DNA-based vectors and retroviral vectors. Methods for constructing and using viral vectors are known in the art [*see, e.g.*, Miller and Rosman, *BioTechniques* 7:980-990 (1992)]. Preferably, the viral vectors are replication defective, that is, they are unable to replicate autonomously in the target cell. In general, the genome of the replication defective viral vectors which are used within the scope of the present invention lack at least one region which is necessary for the replication of the virus in the infected cell. These regions can either be eliminated (in whole or in part), be rendered non-functional by any technique known to a person skilled in the art. These techniques include the total removal, substitution (by other sequences, in particular by the inserted nucleic acid), partial deletion or addition of one or more bases to an essential (for replication) region. Such techniques may be performed *in vitro* (on the isolated DNA) or *in situ*, using the techniques of genetic manipulation or by treatment with mutagenic agents. Preferably, the replication defective virus retains the sequences of its genome which are necessary for encapsulating the viral particles.

DNA viral vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus

(AAV), vaccinia virus, and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. Defective virus is not replication competent after introduction into a cell, and thus does not lead to a productive viral infection. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Thus, a specific tissue can be specifically targeted. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector [Kaplitt et al., *Molec. Cell. Neurosci.* 2:320-330 (1991)], defective herpes virus vector lacking a glyco-protein L gene [Patent Publication RD 371005 A], or other defective herpes virus vectors [International Patent Publication No. WO 94/21807, published September 29, 1994; International Patent Publication No. WO 92/05263, published April 2, 1994]; an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al. [*J. Clin. Invest.* 90:626-630 (1992); *see also* La Salle et al., *Science* 259:988-990 (1993)]; and a defective adeno-associated virus vector [Samulski et al., *J. Virol.* 61:3096-3101 (1987); Samulski et al., *J. Virol.* 63:3822-3828 (1989); Lebkowski et al., *Mol. Cell. Biol.* 8:3988-3996 (1988)].

Preferably, for *in vivo* administration, an appropriate immunosuppressive treatment is employed in conjunction with the viral vector, *e.g.*, adenovirus vector, to avoid immuno-deactivation of the viral vector and transfected cells. For example, immunosuppressive cytokines, such as interleukin-12 (IL-12), interferon-γ (IFN-γ), or anti-CD4 antibody, can be administered to block humoral or cellular immune responses to the viral vectors [*see, e.g.*, Wilson, *Nature Medicine* (1995)]. In addition, it is advantageous to employ a viral vector that is engineered to express a minimal number of antigens.

Naturally, the invention contemplates delivery of a vector that will express a therapeutically effective amount of a CaSR, or an antisense thereto, for gene therapy applications. The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 15 percent, preferably by at least 50 percent, more preferably by at least 90 percent, and most preferably prevent, a clinically significant deficit in the activity, function and response of the host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the host.

Any vector, viral or non-viral, of the invention will preferably be introduced *in vivo* in a pharmaceutically acceptable vehicle or carrier. The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum,

animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

Adenovirus vectors

In a preferred embodiment, the vector is an adenovirus vector. Adenoviruses are eukaryotic DNA viruses that can be modified to efficiently deliver a nucleic acid of the invention to a variety of cell types. Various serotypes of adenovirus exist. Of these serotypes, preference is given, within the scope of the present invention, to using type 2 or type 5 human adenoviruses (Ad 2 or Ad 5) or adenoviruses of animal origin (see WO94/26914). Those adenoviruses of animal origin which can be used within the scope of the present invention include adenoviruses of canine, bovine, murine (example: Mav1, Beard et al., Virology 75 (1990) 81), ovine, porcine, avian, and simian (example: SAV) origin. Preferably, the adenovirus of animal origin is a canine adenovirus, more preferably a CAV2 adenovirus (e.g. Manhattan or A26/61 strain (ATCC VR-800), for example).

Preferably, the replication defective adenoviral vectors of the invention comprise the ITRs, an encapsidation sequence and the nucleic acid of interest. Still more preferably, at least the E1 region of the adenoviral vector is non-functional. The deletion in the E1 region preferably extends from nucleotides 455 to 3329 in the sequence of the Ad5 adenovirus (PvuII-BglII fragment) or 382 to 3446 (HinfII-Sau3A fragment). Other regions may also be modified, in particular the E3 region (WO95/02697), the E2 region (WO94/28938), the E4 region (WO94/28152, WO94/12649 and WO95/02697), or in any of the late genes L1-L5.

In a preferred embodiment, the adenoviral vector has a deletion in the E1 region (Ad 1.0). Examples of E1-deleted adenoviruses are disclosed in EP 185,573, the contents of which are incorporated herein by reference. In another preferred embodiment, the adenoviral vector has a deletion in the E1 and E4 regions (Ad 3.0). Examples of E1/E4-deleted adenoviruses are disclosed in WO95/02697 and WO96/22378, the contents of which are incorporated herein by reference. In still another preferred embodiment, the adenoviral vector has a deletion in the E1 region into which the E4 region and the nucleic acid sequence are inserted (see FR94 13355, the contents of which are incorporated herein by reference).

The replication defective recombinant adenoviruses according to the invention can be prepared by any technique known to the person skilled in the art (Levrero et al., Gene 101 (1991) 195, EP 185 573; Graham, EMBO J. 3 (1984) 2917). In particular, they can be prepared by homologous recombination between an adenovirus and a plasmid which carries, inter alia, the DNA sequence of interest. The homologous recombination is effected following cotransfection of the adenovirus and plasmid into an appropriate cell line. The cell line which is employed should preferably (i) be transformable by the said elements, and (ii) contain the sequences which are able to complement the part of the genome of the

replication defective adenovirus, preferably in integrated form in order to avoid the risks of recombination. Examples of cell lines which may be used are the human embryonic kidney cell line 293 (Graham et al., J. Gen. Virol. 36 (1977) 59) which contains the left-hand portion of the genome of an Ad5 adenovirus (12%) integrated into its genome, and cell lines which are able to complement the E1 and E4 functions, as described in applications WO94/26914 and WO95/02697. Recombinant adenoviruses are recovered and purified using standard molecular biological techniques, which are well known to one of ordinary skill in the art.

Adeno-associated virus vectors

The adeno-associated viruses (AAV) are DNA viruses of relatively small size which can integrate, in a stable and site-specific manner, into the genome of the cells which they infect. They are able to infect a wide spectrum of cells without inducing any effects on cellular growth, morphology or differentiation, and they do not appear to be involved in human pathologies. The AAV genome has been cloned, sequenced and characterised. It encompasses approximately 4700 bases and contains an inverted terminal repeat (ITR) region of approximately 145 bases at each end, which serves as an origin of replication for the virus. The remainder of the genome is divided into two essential regions which carry the encapsulation functions: the left-hand part of the genome, which contains the rep gene involved in viral replication and expression of the viral genes; and the right-hand part of the genome, which contains the cap gene encoding the capsid proteins of the virus.

The use of vectors derived from the AAVs for transferring genes *in vitro* and *in vivo* has been described (see WO 91/18088; WO 93/09239; US 4,797,368, US 5,139,941, EP 488 528). These publications describe various AAV-derived constructs in which the rep and/or cap genes are deleted and replaced by a gene of interest, and the use of these constructs for transferring the said gene of interest in vitro (into cultured cells) or in vivo, (directly into an organism). The replication defective recombinant AAVs according to the invention can be prepared by cotransfecting a plasmid containing the nucleic acid sequence of interest flanked by two AAV inverted terminal repeat (ITR) regions, and a plasmid carrying the AAV encapsulation genes (rep and cap genes), into a cell line which is infected with a human helper virus (for example an adenovirus). The AAV recombinants which are produced are then purified by standard techniques.

The invention also relates, therefore, to an AAV-derived recombinant virus whose genome encompasses a sequence encoding a nucleic acid encoding a CaSR flanked by the AAV ITRs. The invention also relates to a plasmid encompassing a sequence encoding a nucleic acid encoding a CaSR flanked by two ITRs from an AAV. Such a plasmid can be used as it is for transferring the nucleic acid sequence, with the plasmid, where appropriate, being incorporated into a liposomal vector (pseudo-virus).

Retrovirus vectors

In another embodiment the gene can be introduced in a retroviral vector, *e.g.*, as described in

Anderson et al., U.S. Patent No. 5,399,346; Mann et al., 1983, Cell 33:153; Temin et al., U.S. Patent No. 4,650,764; Temin et al., U.S. Patent No. 4,980,289; Markowitz et al., 1988, J. Virol. 62:1120; Temin et al., U.S. Patent No. 5,124,263; EP 453242, EP178220; Bernstein et al. Genet. Eng. 7 (1985) 235; McCormick, BioTechnology 3 (1985) 689; International Patent Publication No. WO 95/07358, published March 16, 1995, by Dougherty et al.; and Kuo et al., 1993, Blood 82:845. The retroviruses are integrating viruses which infect dividing cells. The retrovirus genome includes two LTRs, an encapsulation sequence and three coding regions (gag, pol and env). In recombinant retroviral vectors, the *gag*, *pol* and *env* genes are generally deleted, in whole or in part, and replaced with a heterologous nucleic acid sequence of interest. These vectors can be constructed from different types of retrovirus, such as, HIV, MoMuLV ("murine Moloney leukaemia virus" MSV ("murine Moloney sarcoma virus"), HaSV ("Harvey sarcoma virus"); SNV ("spleen necrosis virus"); RSV ("Rous sarcoma virus") and Friend virus. Defective retroviral vectors are disclosed in WO95/02697.

In general, in order to construct recombinant retroviruses containing a nucleic acid sequence, a plasmid is constructed which contains the LTRs, the encapsulation sequence and the coding sequence. This construct is used to transfect a packaging cell line, which cell line is able to supply in trans the retroviral functions which are deficient in the plasmid. In general, the packaging cell lines are thus able to express the gag, pol and env genes. Such packaging cell lines have been described in the prior art, in particular the cell line PA317 (US4,861,719); the PsiCRIP cell line (WO90/02806) and the GP+envAm-12 cell line (WO89/07150). In addition, the recombinant retroviral vectors can contain modifications within the LTRs for suppressing transcriptional activity as well as extensive encapsulation sequences which may include a part of the gag gene (Bender et al., J. Virol. 61 (1987) 1639). Recombinant retroviral vectors are purified by standard techniques known to those having ordinary skill in the art.

Retroviral vectors can be constructed to function as infections particles or to undergo a single round of transfection. In the former case, the virus is modified to retain all of its genes except for those responsible for oncogenic transformation properties, and to express the heterologous gene. Non-infectious viral vectors are prepared to destroy the viral packaging signal, but retain the structural genes required to package the co-introduced virus engineered to contain the heterologous gene and the packaging signals. Thus, the viral particles that are produced are not capable of producing additional virus.

Targeted gene delivery is described in International Patent Publication WO 95/28494, published October 1995.

Non-viral vectors

Alternatively, the vector can be introduced *in vivo* by lipofection. For the past decade, there has been increasing use of liposomes for encapsulation and transfection of nucleic acids *in vitro*. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome mediated transfection can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker

[Felgner, et. al., *Proc. Natl. Acad. Sci. U.S.A.* **84**:7413-7417 (1987); *see* Mackey, et al., *Proc. Natl. Acad. Sci. U.S.A.* **85**:8027-8031 (1988); Ulmer et al., *Science* **259**:1745-1748 (1993)]. The use of cationic lipids may promote encapsulation of negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes [Felgner and Ringold, *Science* 337:387-388 (1989)]. Particularly useful lipid compounds and compositions for transfer of nucleic acids are described in International Patent Publications WO95/18863 and WO96/17823, and in U.S. Patent No. 5,459,127. The use of lipofection to introduce exogenous genes into the specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. It is clear that directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, such as pancreas, liver, kidney, and the brain. Lipids may be chemically coupled to other molecules for the purpose of targeting [*see* Mackey, et. al., *supra*]. Targeted peptides, *e.g.*, hormones or neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

Other molecules are also useful for facilitating transfection of a nucleic acid *in vivo*, such as a cationic oligopeptide (*e.g.*, International Patent Publication WO95/21931), peptides derived from DNA binding proteins (*e.g.*, International Patent Publication WO96/25508), or a cationic polymer (*e.g.*, International Patent Publication WO95/21931).

It is also possible to introduce the vector *in vivo* as a naked DNA plasmid. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter [*see, e.g.*, Wu et al., *J. Biol. Chem.* **267**:963-967 (1992); Wu and Wu, *J. Biol. Chem.* **263**:14621-14624 (1988); Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990; Williams et al., *Proc. Natl. Acad. Sci. USA* **88**:2726-2730 (1991)]. Receptor-mediated DNA delivery approaches can also be sued [Curiel et al., *Hum. Gene Ther.* **3**:147-154 (1992); Wu and Wu, *J. Biol. Chem.* 262:4429-4432 (1987)].

This invention provides several embodiments for specifically inhibiting CaSR activity in a patient suffering from a disease or disorder associated with abnormal calcium levels.

As a first embodiment, CaSR expression is inhibited by nucleic acids comprising a sequence complementary to the sequence encoding CaSR, or isoform thereof, and down-regulating or blocking its expression. A preferred embodiment comprises an antisense polynucleotide molecule. Preparation and use of antisense polynucleotides, DNA encoding antisense RNA molecules and use of oligo and genetic antisense is disclosed in WO 92/15680, the entire contents of which are incorporated herein by reference.

Antisense nucleic acids of the invention are preferably RNA capable of specifically hybridizing with all or part of the sequence selected from the group consisting of SEQ ID No. 7, SEQ ID No. 9, and SEQ ID No. 11 or the corresponding messenger RNA. The antisense sequence of the present invention may be derived from DNA sequences whose expression in the cell produces RNA

complementary to all or part of the CaSR. These antisense sequences can be prepared by expression of all or part of the sequence selected from the group consisting of SEQ ID No. 7, SEQ ID No. 9, and SEQ ID No. 11 in the opposite orientation (EP 140 308). Any length of the antisense sequence is suitable for practice of the invention so long as it is capable of down-regulating or blocking expression of the CasR, or isoform thereof. Preferably, the antisense sequence is at least 20 nucleotides in length.

In another aspect of this preferred embodiment the nucleic acid encodes antisense RNA molecules. In this embodiment, the nucleic acid is operably linked to signals enabling expression of the nucleic acid sequence, and is introduced into a cell utilizing, preferably, recombinant vector constructs, which will express the antisense nucleic acid once the vector is introduced into the cell. Examples of suitable vectors includes plasmids, adenoviruses, adeno-associated viruses, retroviruses, and herpes viruses as described above.

Suitable expression signals include transcriptional promoter and termination sequences. Among the promoter sequences useful for practice of this invention are tetracycline-regulated transcriptional modulators and CMV, SV-40, E1a, MLP, and LTR promoters. Tetracycline-regulated transcriptional modulators and CMV promoters are described in WO 96/01313, US 5,168,062 and 5,385,839, the entire contents of which are incorporated herein by reference. The nucleic acid constructs of this invention are capable of down-regulating or blocking expression of a CaSR, or isoform thereof, and are delivered, in a preferred aspect of the invention, locally to cells capable of regulating calcium levels in a patient.

A second embodiment of the present invention's method of specifically inhibiting human CaSR activity, or an isoform thereof, at selected sites, comprises inhibiting CaSR function by expression of a nucleic acid sequence encoding an intracellular binding protein capable of selectively interacting with the CaSR, or isoform thereof, within a transfected cell. WO 94/29446 and WO 94/02610, the entire contents of which are incorporated herein by reference, disclose cellular transfection with genes encoding an intracellular binding protein. An intracellular binding protein includes any protein capable of selectively interacting, or binding, with a CaSR, or isoform thereof, in the cell in which it is expressed and of neutralizing the function of bound CaSR. Preferably, the intracellular binding protein is an antibody or a fragment of an antibody. More preferably, the antibody or fragment thereof binds the cytoplasmic domain of the CaSR. Most preferably, the intracellular binding protein is a single chain antibody capable of inhibiting cellular calcium sensing.

WO 94/02610 discloses preparation of antibodies and identification of the nucleic acid encoding a particular antibody. Using the CaSR or an isoform thereof, a monoclonal antibody specific for the cytoplasmic domain is prepared by according to techniques known to those skilled in the art. A vector comprising the nucleic acid encoding an intracellular binding protein, or a portion thereof, and capable of expression in a host cell is subsequently prepared for use in the method of this invention. Suitable vectors and methods of delivering nucleic acids encoding intracellular binding proteins to cells containing a CaSR include those discussed above for delivery of antisense nucleic

acids.

In a preferred aspect of this second embodiment, the nucleic acid sequence encoding a CaSR intracellular binding protein additionally comprises a sequence encoding a localization signal for targeting the intracellular binding protein to the cellular location of CaSR and/or a sequence enabling insertion of the intracellular binding protein in the plasma membrane. The localization signal or insertion sequence can be located anywhere on the intracellular binding protein, so long as it does not interfere with binding to the CaSR or isoform thereof. Examples of localization signals are disclosed in WO 94/02610. Preferably, the localization signal targets the intracellular binding protein to the plasma membrane.

The present invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention.

EXAMPLES

Material and Methods

General Materials and Methods:

Bacterial strain. The strain TG1 of *Escherichia coli* of the genotype supE, hsdD5, thi, D(lac-proAB), F'[tra D36 pro A^+B^+ $lacI^q$ lacZDM15] may be used as a means to amplify and isolate the recombinant plasmids utilized.

It may be cultivated on:

LB medium: -NaCl (5 g/l) (Difco)
-Bacto-tryptone (10 g/l) (Difco)
-Yeast extract (5 g/l) (Difco)

This medium is rendered solid by the addition de 20 g/l of agar (Difco). Ampicillin (100 µg/ml) permits selection of the bacteria that have received the plasmids that carry the gene imparting resistance to this antibiotic as a marker.

Plasmids.

Bluescript series vectors (Stratagene), may used. These vectors permit cloning to be performed just like the pMTL series (Chambers et al.; Gene 1988, 68, pp 139-149).

In addition, the vectors pCDNA3 (Invitrogen) and derivative vectors (pSG42 and pCNW8), which permit the expression of proteins in mammal cells under the control of the CMV promoter, may be used.

Also, the vectors pCRII or pCR2.1 (Invitrogen), which permit cloning of PCR fragments, may be used.

The genetic engineering techniques used to clone and insert cDNAs into these plasmids employ routine protocols (Maniatis T. et al., "Molecular Cloning, a Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982; Ausubel F.M. et al. (eds.), "Current Protocols in Molecular Biology," John Wiley & Sons, New York, 1987).

Preparation of the plasmid DNA. Large quantities of DNA may be prepared using Promega's rapid DNA preparation kit in accordance with the manufacturer's instructions. Small quantities of DNA may be prepared in the following manner: bacteria containing the plasmid are cultivated for at least 4 hours in 2 ml of LB medium in a shaker with agitation. They are then centrifuged for 2 minutes at 14,000 rpm in Eppendorf tubes, then the concentrate is put back in suspension in 100 µl of solution I (50 mM of glucose, 25 mM of Tris-HCl pH 8 buffer, 10 mM of EDTA pH 8), lysed with 200 µl of solution II (0.2 M of NaOH, 1% SDS). The lysis solution is then neutralized with 150 µl of solution III (3 M of potassium acetate, 11.5% (v/v) glacial acetic acid). After agitation of the tubes until a flocculent precipitate is obtained, 150 µl of a mixture of phenol/chloroform (50% phenol and 50% chloroform saturated in water) is added, and the entire mixture is agitated for 30 seconds. The aqueous phase containing the DNA is recovered after centrifugation for 2 minutes at 14,000 rpm. The DNA is then precipitated via the addition of 0.5 volume of isopropanol, then centrifuged for 5 minutes at 14,000 rpm and air-dried in order to finally be dissolved in 20 µl of TE-RNAse (solution of 10 mM of Tris-HCl and 1 mM of EDTA with 50 µg/ml of RNAse).

Enzyme amplification of DNA by Polymerase Chain Reaction (PCR). PCR reactions may be carried out in a final volume of 100 µl in the presence of the double stranded DNA, dNTP (0.2 mM), PCR buffer (10 mM of Tris-HCL pH 8.5, 1 mM of $MgCl_2$, 5 mM of KCl, gelatin 0.01%), 0.5 µg of each of the oligonucleotides, and 2.5 IU of Ampli Taq DNA polymerase (Perkin Elmer) with or without formamide (5%). The mixture iss covered with 2 drops of paraffin oil to limit evaporation of the sample. The equipment used may be Appligene's "Crocodile II." Unless otherwise specified, denaturation is effected at a temperature of 90°C for denaturation of the helix, a temperature for hybridization of the oligonucleotides to the denatured (single-stranded) DNA that iss 5 to 10 degrees lower than the temperature for the separation of the oligonucleotides, and a temperature of 72°C for elongation by the enzyme. The fragments obtained by PCR, which are used for cloning, are systematically resequenced once they were cloned, so as to verify the absence of any mutations that might have occurred during the amplification.

The oligodeoxynucleotides may be chemically synthesized according to the phosphoramidite method by utilizing ß-cyanoethyl protector groups. After synthesis, the protector groups are eliminated by treatment with ammonia, and two precipitations with butanol permit purification and concentration of the oligodeoxynucleotides. The DNA concentration may be determined by measuring the optical density at 260 nm.

Ligations. Ligation reactions may be carried out at +14°C for one night in a final volume of 10 µl in the presence of 100 to 200 ng of vector, 0.5 to 2 µg of insert, 40 IU of enzyme T4 DNA ligase (Biolabs), and a ligation buffer (50 mM of Tris-HCl pH 7.8; 10 mM of $MgCl_2$; 10 mM of DTT; 1 mM of ATP). The negative control is formed by the ligation of the vector in the absence of the insert.

The filling of the prominent 5' ends is carried out, as needed, before ligation via the Klenow fragment of DNA Polymerase I of *E. coli* (Biolabs) according to the supplier's specifications. The destruction of the prominent 3' ends is accomplished in the presence of DNA Polymerase of the T4 phage (Biolabs) used according to the manufacturer's recommendations.

Transformation of bacteria. The entire ligation volume (10 µl) may be used to transform bacteria, which may be rendered competent by the method of Chung et al. (1988, Proc. Natl. Acad. Sci. 86:2172-2175). The bacteria are placed in culture in a liquid LB medium for several hours in an incubator with agitation at 37°C until an OD of 0.6 was obtained at 600 nm. The medium is then centrifuged at 6,000 rpm for 10 mn. The bacteria are rendered competent by dissolving the bacterial concentrate in a volume of TSB (LB medium + 100 g/l of PEG 4000, 5% DMSO, 10 mM of $MgCl_2$, 10 mM of $MgSO_4$) corresponding to 1/10 of the volume of the medium of the initial culture. After incubation at 4°C for 30 to 60 minutes, 200 µl of bacteria are placed in contact with the ligation products for 15 minutes on ice. After the addition of 200 µl of LB [medium], the bacteria are incubated for 30 mn at 37°C, then spread out on an LB + ampicillin medium.

Separation and extraction of the DNA. The separation of the DNA is performed by electrophoresis as a function of their size. In order to do this, different gels are used depending on the size of the fragments to be separated:

-1% agarose gel (Gibco BRL) in a TBE buffer (90 mM of Tris base; 90 mM of borate; 2 mM of EDTA) to separate large DNA fragments (greater than 500 bp);

-2% NuSieve agarose gel (FMC Bioproducts) in a TBE buffer to separate small fragments (less than 500 bp).

Migration on agarose gel or on polyacrylamide gel is carried out in a TBE buffer and in the presence of a molecular weight marker (1 Kb ladder, Gibco BRL). The DNA was mixed with 1/10 of the deposit volume of blue (200 g/l of Ficoll, 0.5 g/l of bromophenol blue, 50 mM of EDTA) before being deposited on the gel. After migration at 100 Volts and staining with ethidium bromide (concentration 0.5 µg/ml of gel), the bands are viewed under a UV lamp.

Extraction of the DNA from the band of an agarose gel is carried out by means of electroelution as follows: the piece of gel containing the DNA fragment s cut out with a scalpel and placed in a dialysis tube closed with two clamps and containing 100 to 500 µl of TBE. The entire mixture is placed in an electrophoresis tank, where it is subjected to an electrical field of 100 Volts. After being removed from the gel, the DNA is then purified by means of two extractions with phenol/chloroform followed by two

extractions with chloroform, then precipitated in the presence of 0.3 M of sodium acetate and 2.5 volume of absolute alcohol. After centrifugation (5 mn at 14,000 rpm), the DNA concentrate is dried and then dissolved in 20 µl of water.

Fluorescent sequencing of plasmid DNA. The sequencing may be carried out according to Sanger's method using 4 dideoxyribonucleotides possessing a different fluorescent marker. The incorporation of one of these dideoxyribonucleotides causes a halt in the replication by the polymerase Taq of the DNA to be sequenced. This reaction yields DNA fragments of various sizes, all of which are terminated at 3' by one of the 4 dideoxyribonucleotides. One µg of a plasmid and 4 picomoles of a primer are added to 9.5 µl of a "premix" supplied by Applied Biosystems under the trademark PRISM©. The final volume is 20 µl in order to perform a PCR for 25 cycles, broken down into a denaturation phase at 96°C for 30 seconds, a hybridization phase at 50°C for 15 seconds, and an elongation phase at 60°C for 4 minutes. DNA fragments obtained after amplification are purified on an exclusion column (Chromaspin-30 from Clontech) and are then dried in a Speed Vac. All of the dried material is dissolved in 5 µl of a mixture made up of 24 µl of EDTA (50 mM) and 120 µl of deionized formamide. After denaturation at 96°C for 3 minutes, 3 to 5 µl are deposited on an electrophoresis gel. The different DNA fragments are separated according to their size and then successively passed in front of a laser reader of the ABI 370 DNA sequencer (Applied Biosystems), where the different fluorescent chromophores are detected.

EXAMPLE 1: PCR amplification of CaSR splice variants

First-strand cDNA, isolated from total RNA from normal human adult tissue, was purchased from Invitrogen. The RNA was subsequently treated with DNase (RNase-free) to eliminate genomic DNA contamination. Ten micrograms (10ug) of the RNA is primed with an Oligo (dT) primer and reverse transcribed with MMLV reverse transcriptase. The reaction is stopped by incubating at 65°C for 10 minutes. The cDNA is in 40ul of RT buffer. (1xRT Buffer: 50mM Tris HCl, pH 8.3, 75 mM KCl, 3mM MgCl2, 10mM DTT).

The following oligonucleotide primers were used to identify CaSR isoforms:

Primer	Sequence	Wild type CaSR position	
1-AS	5'-CATGGGTCAATTCACTGTCAT-3'	3383 - 3403	(SEQ ID NO:1)
3-AS	5'-GCCAGATCACACAGATGACAA-3'	2207 - 2227	(SEQ ID NO:2)
4-AS	5'-GGCATAGACGTTGTAATACCC-3'	1525 - 1545	(SEQ ID NO:3)
5-AS	5'-TGTGGACAGACTTCCTGGGAT-3'	1013 - 1033	(SEQ ID NO:4)
5-S	5'-ATCCCAGGAAGTCTGTCCACA-3'	1013 - 1033	(SEQ ID NO:5)
7-S	5'-ACTCCTAGCTGTCTCATCCCT-3'	-44 - -24	(SEQ ID NO:6)

Splice variant CaSRc was amplified with Perkin Elmer's AmpliTaq Gold. The final reaction mix consisted of 10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM MgCl2, 0.001 (w/v) gelatin, 0.8mM dATP, 0.8mM dCTP, 0.8mM dGTP, 0.8mM dTTP, 2.5 Units AmpliTaq Gold, 0.4uM Primer 3-AS, 0.4uM Primer 5-S, and 2ul Invitrogen human kidney cDNA / 100ul reaction. Reaction conditions are: hold at 95°C for 9 minutes; 40 cycles of 94°C - 30 seconds, 60°C - 1 minute; and hold at 60°C for 10 minutes.

CaSRb was amplified using the same conditions as CaSRc, except that 0.4uM Primer 5-AS and 0.4uM Primer 7-S were used in place of Primers 3-AS and 5-S.

Splice variant CaSRd was amplified using the same components as CaSRc with the following exceptions: 0.4uM Primer 1-AS and 0.4uM Primer 7-S were used in place of Primers 3-AS and 5-S, and 3ul instead of 2ul of Invitrogen human kidney cDNA / 100ul reaction was used. Reaction conditions were: hold at 95°C for 9 minutes; 20 cycles of 94°C - 30 seconds, 64°C - 3 minutes; 20 cycles of 94°C - 30 seconds, 64°C - 3 minutes (increment 10 seconds / cycle); hold at 60°C for 10 minutes. 50ul of this reaction mix was then electrophoresed on a 1% agarose gel and the bands in the 3.0 - 3.4kb region were excised and extracted using Qiagen's Gel Extraction kit and eluted in 50ul ddH2O. 1ul of this extract was used as a template for the next reaction which contained the same components as the previous reaction with the following exceptions: 0.4uM Primer 4-AS was used instead of Primer 1-AS, and the template was replaced. Reaction conditions for the first and second

amplifications were identical.

All PCR products were cloned into pCR2.1 according to Invitrogen's protocols. Sequencing was performed with an automated DNA sequencer.

EXAMPLE 2: Expression of CaSRb and CaSRc in human kidney

The strategy of searching for CaSR splice variants involved the use of primer pairs to scan different parts of of the sensor cDNA. Human kidney first strand cDNA was used as template and was amplified with either primer pair 3AS/5S or 5AS/7S. Electrophoretic resolution of the PCR mixture obtained with primer pair 3AS/5S revealed the presence of a product with the expected size (1.2kb) of the wild type CaSR and one with lower molecular mass of approximately 1.0kb. The primer pair 5AS/7S also yielded two visible PCR products with estimated size of 1.0 and 0.7kb. The 1.0kb product corresponded to the expected size of the wild type CaSR. The PCR products from both primer pairs were ligated into pCR 2.1 and multiple clones were selected for analysis by restriction digestion with EcoR1 to release the insert. Clones bearing the putative wild type CaSR insert and those with smaller insert size were sequenced. The results of these experiments confirmed the presence of the correct CaSR sequence in the putative wild type clones. On the other hand, clones with shorter insert were found to contain either a deletion from nucleotide 186-495 or from nucleotide 1378-1608. The 186-495 deleted CaSR corresponds to CaSRb described originally in medullary thyroid carcinoma. The 1378-1608 deleted CaSR is designated as CaSRc (Figure 1). Unlike CaSRb, the deletion in CaSRc does not cause a shift in reading frame.

Example 3: Expression of CaSRd in human kidney

Following an initial PCR enrichment of human kidney first strand cDNA with primer pair 1AS/7S, products in the 3.0 - 3.4 kb range were gel purified and were amplified further with primer pair 4AS/7S. The PCR products were isolated by TA cloning and sequenced. One of these clones was found to contain a deletion from nucleotide 1075-1386 (Figure 1). The 1075-1386 deleted CaSR is designated CaSRd. No change in reading frame was detected in this alternatively spliced CaSR transcript.

Example 4: Stable expression of isoform CaSRd in HEK-293 cells

Full length CaSRd was cloned into the mammalian expression vector pCEP4 (from Invitrogen). The CaSRd DNA used for transfection was prepared using the Qiagen plasmid

preparation kit. LipofectAMINE (Life Technologies, Inc.) was used as a carrier for transfection. Transfection of HEK-293 cells with CaSRd DNA was performed according to the general protocol described in the LipofectAMINE transfection kit. The CaSRd DNA and lipofectamine complex (1 ml) was overlayed onto HEK-293 cells (90% confluent) in 6 well plates. After 5 hr. at 37°C, 1 ml of DMEM containing 20% fetal bovine serum, penicillin and streptomycin was added to each well. After incubating at 37°C for 16 hours, the media was replaced with 2 ml of DMEM containing 10% bovine serum albumin, penicillin and streptomycin and the cultures were incubated further for 8 hours at 37°C. CaSRd transfectants were isolated by selection in the presence of hygromycin following limited dilution. The cells in each well were trypsinized, and cultures were diluted in 100 ml of DMEM containing 10% fetal bovine serum, 200 ug/ml of hygromycin, penicillin and streptomycin. 1 ml aliquots of the diluted cultures were added to each well of several 24 well tissue culture plates. After 4 weeks in culture, wells containing a single colony were identified and each cell clone was expanded into a T75 flask. The expression of CaSRd in each cell clone was monitored by Northern analysis. A clone, 21/2 with the highest expression level was used for functional analysis as described below.

The function of isoform CaSRd was assayed by its ability to increase intracellular concentration in response to elevation in extracellular calcium concentrations and other agonists. The wild type receptor has been shown to increase intracellular calcium concentration when extracellular calcium concentrations were raised. Intracellular calcium was measured with the fluorescent indicator, fura-2 (from Molecular Probes). HEK-293 cells transfected with CaSRd was loaded in buffer containing 0.5uM fura-2, 20mM HEPES, pH 7.35, 0.1% BSA, 0.5mM $CaCl_2$, 0.5mM $MgCl_2$, 6.7mM KCl, 3mM glucose and 142mM NaCl for 45 min at 37°C. The cells were washed and resuspended to 2 x 10^6 cells/ml in the loading buffer without fura-2. For intracellular calcium measurement, cells were placed in a quartz cuvette equilibrated at 37°C. Excitation monochrometers were centered at 340 and 380 nm with emission light collected at 505 nm. Different CaSR agonists were added to different final concentrations to activate the CaSR. Usually a final concentration of 10mM external $CaCl_2$ is sufficient to activate the wild type receptor maximally. Results indicated that CaSRd did not respond to agonists such as Ca^{++}, Mg^{++} and neomycin but did respond to gadolinium (Table I). In the presence of a CaSR potentiating compound, NPS568 (WO 94/18959, Fox et al., (1993) J. Bone Min. Res. 8: S181; Abstract #260) CaSRd responded to calcium, magnesium and neomycin.

Table I

Effects of extracellular Ca^{++}, NPS568 and gad linium on intracellular calcium level in CaSRd expressing HEK-293 cells

Increase in fura-2 fluorescence (cps x 10^6)

	Expt 1	Expt 2
20mM Ca^{2+}	0.2	0
20mM Ca^{2+} + 2uM NPS568	1.5	1.7
10uM NPS568	0.2	0.1
100uM gadolinium	2.0	

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid encoding an isoform of a human calcium sensing receptor, wherein the nucleic acid comprises about 2922 to about 3003 nucleotides and has a deletion of about 231 nucleotides when compared to the wild-type form of the receptor as depicted in SEQ ID NO:11.

2. The nucleic acid according to claim 1, wherein said deletion is from the region encoding the extracellular domain of the receptor.

3. The nucleic acid according to claim 2, wherein the deletion is from about nucleotides 1075-1386 of SEQ ID NO:11.

4. The nucleic acid according to claim 2, wherein the deletion is from about nucleotides 1378-1608 of SEQ ID NO:11.

5. The nucleic acid according to claim 2, wherein the deletion is from about nucleotides 1075-1608 of SEQ ID NO:11.

6. The nucleic acid according to claim 1, having at least one property selected from

   a) it can be amplified by polymerase chain reaction (PCR) using an oligonucleotide primer derived from SEQ ID NO:7, SEQ ID NO:9 or SEQ ID NO:11;

   b) it hybridizes under stringent conditions with a nucleic acid having a nucleotide sequence as depicted in SEQ ID NO:7 or SEQ ID NO:9; and

   c) it encodes a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:10, and allelic variants thereof.

7. The isolated nucleic acid of claim 2, wherein the isoform comprises an amino acid sequence as depicted in SEQ ID NO:8 or SEQ ID NO:10, or allelic variants thereof.

8. The isolated nucleic acid of claim 2 comprising a nucleotide sequence as depicted in SEQ ID NO:7 or SEQ ID NO:9, or allelic variants thereof.

9. The isolated nucleic acid of claim 1 wherein the nucleic acid can be amplified by polymerase chain reaction (PCR) using an oligonucleotide primer selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:6.

10. A vector comprising the nucleic acid of claim 1.

11. The vector according to claim 10 wherein the nucleic acid is operatively associated with an expression control sequence permitting expression of the receptor in an expression competent host cell.

12. The vector according to claim 11 selected from the group consisting of an RNA molecule, a plasmid DNA molecule, and a viral vector.

13. The vector according to claim 12 which is a plasmid DNA molecule.

14. The vector according to claim 12 which is a viral vector selected from the group consisting of

retrovirus, adenovirus, adeno-associated virus, herpes virus, and vaccinia virus.

15. A host cell transfected with the vector of claim 10.

16. A host cell transfected with the vector of claim 13.

17. The host cell of claim 15 selected from the group consisting of a bacterial cell, a yeast cell, and a mammalian cell.

18. A method for expressing an isoform of human calcium sensing receptor comprising:

a) culturing the host cell of claim 17 in culture medium under conditions permitting expression of the receptor; and

b) identifying cells expressing the receptor on their surface.

19. An isolated isoform of a human calcium sensing receptor, wherein the isoform comprises about 974 to about 1001 amino acids and has a deletion of about 77 amino acids when compared to the wild-type form of the receptor as depicted in SEQ ID NO:12.

20. The isoform according to claim 19, wherein said deletion is from the extracellular domain of the receptor.

21. The isoform according to claim 20, wherein the deletion is from about amino acids 358-462 of SEQ ID NO:12.

22. The isoform according to claim 20, wherein the deletion is from about amino acids 460-536 of SEQ ID NO:12.

23. The isoform according to claim 20, wherein the deletion is from about amino acids 358-536 of SEQ ID NO:12.

24. The isoform of claim 19, wherein the isoform comprises an amino acid sequence as depicted in SEQ ID NO:8 or SEQ ID NO:10, or allelic variants thereof.

25. A method of screening for agonists or antagonists of a CaSR isoform activity, the method comprising incubating a test sample with a CaSR isoform, measuring CaSR isoform activity and comparing the activity to that in the absence of the test sample.

26. The method according to claim 25, wherein the CaSR activity is its ability to influence intracellular calcium concentration.

27. The method according to claim 25, wherein the test sample is tested alone, in conjunction with an elevation in extracellular calcium concentration, or in the presence of other agonists or antagonists of CaSR isoform activity.

28. The method according to claim 26, wherein the intracellular calcium concentration is measured with a fluorescent indicator.

29. The method according to claim 28, wherein the indicator is fura-2.

30. A method of treating a patient suffering from a disease or disorder associated with abnormal calcium levels, the method comprising the administration of a therapeutically effective

amount of a compound capable of modulating the activity of a CaSR isoform.

31. The method according to claim 30, wherein the disease is hyperparathyroidism or osteoporosis.

32. The method according to claim 30, wherein the disease is Paget's disease, hypercalcemia malignancy, or hypertension.

33. The method according to claim 30, wherein the compound is an agonist or an antagonist of a CaSR isoform.

34. A method of increasing or decreasing the level of CaSR activity in a cell, the method comprising administering to the cell a nucleic acid capable of increasing or decreasing CaSR activity.

35. The method according to claim 34, wherein the cells are in a patient suffering from a disease or disorder associated with abnormal calcium levels.

36. The method according to claim 35, wherein the disease is hyperparathyroidism or osteoporosis.

37. The method according to claim 35, wherein the disease is Paget's disease, hypercalcemia malignancy, or hypertension.

38. The method according to claim 35, wherein the nucleic acid is in a vector.

39. The method according to claim 38, wherein the nucleic acid encodes an isoform of a human calcium sensing receptor, wherein the nucleic acid comprises about 2922 to about 3003 nucleotides and has a deletion of about 231 nucleotides when compared to the wild-type form of the receptor as depicted in SEQ ID NO:11.

40. The method according to claim 38, wherein said vector is selected from the group consisting of plasmids, retroviruses, herpes simplex viruses, adeno-associated viruses, adenoviruses, and vaccinia viruses.

41. The method according to claim 27, wherein the nucleic acid encodes an intracellular binding protein capable of binding a CaSR, or an isoform thereof.

42. The method according to claim 41, wherein the intracellular binding protein is an antibody or fragment thereof.

43. The method according to claim 42, wherein the antibody or fragment thereof binds the cytoplasmic domain of a CaSR.

44. The method according to claim 43, wherein the antibody or fragment thereof is a single chain antibody.

45. The method according to claim 41, wherein said nucleic acid further comprises a sequence encoding a localization signal for targeting the intracellular binding protein to the cellular location of a CaSR.

46. The method according to claim 45, wherein said localization signal is specific for the plasma membrane.

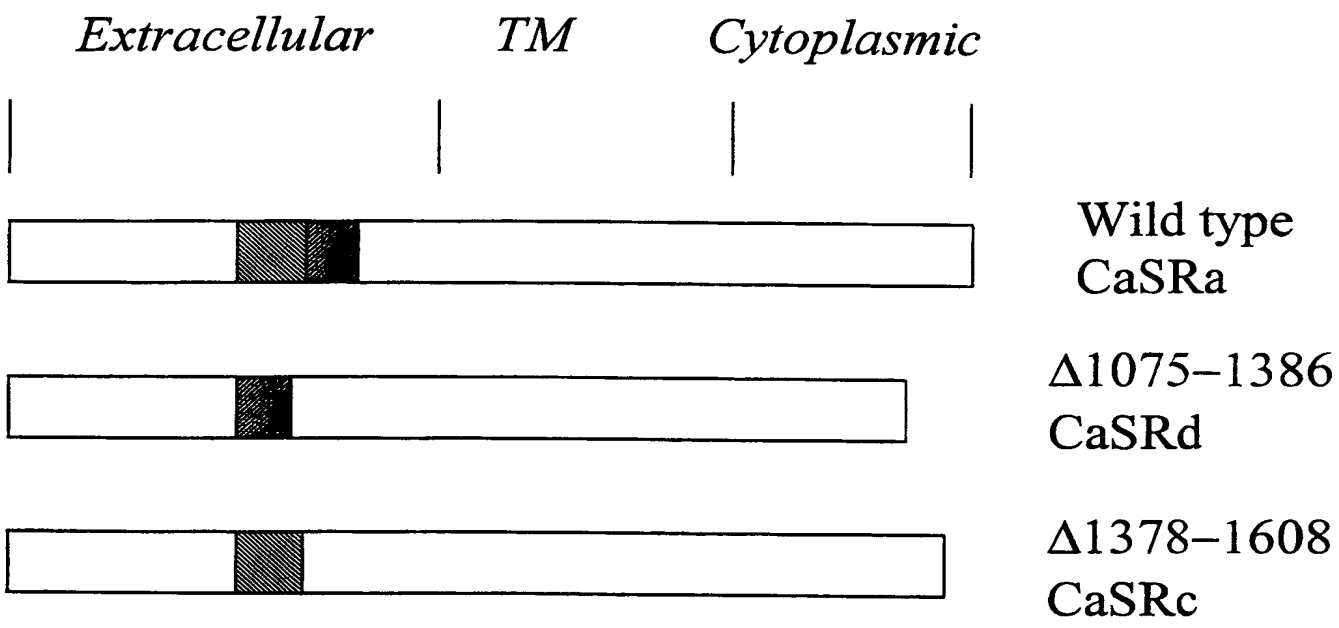
47. The method according to claim 34, wherein said nucleic acid encodes an antisense molecule complementary to the sequence encoding a CaSR and capable of selectively inhibiting the expression of said sequence.

48. The method according to claim 47, wherein said antisense molecule is at least about 20 nucleotides in length.

49. An antigenic polypeptide comprising an an epitope and/or sequence not present in the wild-type CaSR, wherein said polypeptide elicits antibodies which bind to a CaSR isoform.

50. An antibody that specifically recognizes an isoform of CaSR, and does not bind the wild-type CaSR

Figure 1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TM
Cytoplasmic
Wild type CaSRa
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Δ1378–1608 CaSRc

SEQUENCE LISTING

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Thrower, Larry W.
Labaudiniere, Richard F.

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Ser Phe Val Ala Gln Asn Lys Ile Asp Ser Leu Asn Leu Asp Glu Phe
        115                 120                 125

Cys Asn Cys Ser Glu His Ile Pro Ser Thr Ile Ala Val Val Gly Ala
    130                 135                 140

Thr Gly Ser Gly Val Ser Thr Ala Val Ala Asn Leu Leu Gly Leu Phe
145                 150                 155                 160

Tyr Ile Pro Gln Val Ser Tyr Ala Ser Ser Ser Arg Leu Leu Ser Asn
                165                 170                 175

Lys Asn Gln Phe Lys Ser Phe Leu Arg Thr Ile Pro Asn Asp Glu His
            180                 185                 190

Gln Ala Thr Ala Met Ala Asp Ile Ile Glu Tyr Phe Arg Trp Asn Trp
        195                 200                 205

Val Gly Thr Ile Ala Ala Asp Asp Asp Tyr Gly Arg Pro Gly Ile Glu
    210                 215                 220

Lys Phe Arg Glu Glu Ala Glu Glu Arg Asp Ile Cys Ile Asp Phe Ser
225                 230                 235                 240

Glu Leu Ile Ser Gln Tyr Ser Asp Glu Glu Glu Ile Gln His Val Val
                245                 250                 255

Glu Val Ile Gln Asn Ser Thr Ala Lys Val Ile Val Val Phe Ser Ser
            260                 265                 270

Gly Pro Asp Leu Glu Pro Leu Ile Lys Glu Ile Val Arg Arg Asn Ile
        275                 280                 285

Thr Gly Lys Ile Trp Leu Ala Ser Glu Ala Trp Ala Ser Ser Ser Leu
    290                 295                 300

Ile Ala Met Pro Gln Tyr Phe His Val Val Gly Gly Thr Ile Gly Phe
305                 310                 315                 320

Ala Leu Lys Ala Gly Gln Ile Pro Gly Phe Arg Glu Phe Leu Lys Lys
                325                 330                 335

Val His Pro Arg Lys Ser Val His Asn Gly Phe Ala Lys Glu Phe Trp
            340                 345                 350

Glu Glu Thr Phe Asn Cys His Leu Gln Glu Gly Ala Lys Gly Pro Leu
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Pro Val Asp Thr Phe Leu Arg Gly His Glu Glu Ser Gly Asp Arg Phe
370 375 380

Ser Asn Ser Ser Thr Ala Phe Arg Pro Leu Cys Thr Gly Asp Glu Asn
385 390 395 400

Ile Ser Ser Val Glu Thr Pro Tyr Ile Asp Tyr Thr His Leu Arg Ile
405 410 415

Ser Tyr Asn Val Tyr Leu Ala Val Tyr Ser Ile Ala His Ala Leu Gln
420 425 430

Asp Ile Tyr Thr Cys Leu Pro Gly Arg Gly Leu Phe Thr Asn Gly Ser
435 440 445

Cys Ala Asp Ile Lys Lys Val Glu Ala Trp Gln Val Pro Phe Ser Asn
450 455 460

Cys Ser Arg Asp Cys Leu Ala Gly Thr Arg Lys Gly Ile Ile Glu Gly
465 470 475 480

Glu Pro Thr Cys Cys Phe Glu Cys Val Glu Cys Pro Asp Gly Glu Tyr
485 490 495

Ser Asp Glu Thr Asp Ala Ser Ala Cys Asn Lys Cys Pro Asp Asp Phe
500 505 510

Trp Ser Asn Glu Asn His Thr Ser Cys Ile Ala Lys Glu Ile Glu Phe
515 520 525

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530 535 540

Leu Gly Ile Phe Leu Thr Ala Phe Val Leu Gly Val Phe Ile Lys Phe
545 550 555 560

Arg Asn Thr Pro Ile Val Lys Ala Thr Asn Arg Glu Leu Ser Tyr Leu
565 570 575

Leu Leu Phe Ser Leu Leu Cys Cys Phe Ser Ser Ser Leu Phe Phe Ile
580 585 590

Gly Glu Pro Gln Asp Trp Thr Cys Arg Leu Arg Gln Pro Ala Phe Gly
595 600 605

Ile Ser Phe Val Leu Cys Ile Ser Cys Ile Leu Val Lys Thr Asn Arg
610 615 620

Val Leu Leu Val Phe Glu Ala Lys Ile Pro Thr Ser Phe His Arg Lys
625 630 635 640

Trp Trp Gly Leu Asn Leu Gln Phe Leu Leu Val Phe Leu Cys Thr Phe
645 650 655

Met Gln Ile Val Ile Cys Val Ile Trp Leu Tyr Thr Ala Pro Pro Ser
660 665 670

Ser Tyr Arg Asn Gln Glu Leu Glu Asp Glu Ile Ile Phe Ile Thr Cys
675 680 685

His Glu Gly Ser Leu Met Ala Leu Gly Phe Leu Ile Gly Tyr Thr Cys
690 695 700

Leu Leu Ala Ala Ile Cys Phe Phe Phe Ala Phe Lys Ser Arg Lys Leu
705 710 715 720

Pro Glu Asn Phe Asn Glu Ala Lys Phe Ile Thr Phe Ser Met Leu Ile
725 730 735

Phe Phe Ile Val Trp Ile Ser Phe Ile Pro Ala Tyr Ala Ser Thr Tyr
740 745 750

Gly Lys Phe Val Ser Ala Val Glu Val Ile Ala Ile Leu Ala Ala Ser
755 760 765

Phe Gly Leu Leu Ala Cys Ile Phe Phe Asn Lys Ile Tyr Ile Ile Leu
770 775 780

Phe Lys Pro Ser Arg Asn Thr Ile Glu Glu Val Arg Cys Ser Thr Ala
785 790 795 800

Ala His Ala Phe Lys Val Ala Ala Arg Ala Thr Leu Arg Arg Ser Asn
805 810 815

Val Ser Arg Lys Arg Ser Ser Ser Leu Gly Gly Ser Thr Gly Ser Thr
820 825 830

Pro Ser Ser Ser Ile Ser Ser Lys Ser Asn Ser Glu Asp Pro Phe Pro
835 840 845

Gln Pro Glu Arg Gln Lys Gln Gln Gln Pro Leu Ala Leu Thr Gln Gln
850 855 860

Glu Gln Gln Gln Gln Pro Leu Thr Leu Pro Gln Gln Gln Arg Ser Gln
865 870 875 880

Gln Gln Pro Arg Cys Lys Gln Lys Val Ile Phe Gly Ser Gly Thr Val
885 890 895

Thr Phe Ser Leu Ser Phe Asp Glu Pro Gln Lys Asn Ala Met Ala His
900 905 910

Arg Asn Ser Thr His Gln Asn Ser Leu Glu Ala Gln Lys Ser Ser Asp
915 920 925

Thr Leu Thr Arg His Gln Pro Leu Leu Pro Leu Gln Cys Gly Glu Thr
930 935 940

Asp Leu Asp Leu Thr Val Gln Glu Thr Gly Leu Gln Gly Pro Val Gly
945 950 955 960

Gly Asp Gln Arg Pro Glu Val Glu Asp Pro Glu Glu Leu Ser Pro Ala
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Thr Ser Ala Tyr Gly Pro Asp Gln Arg Ala Gln Lys Lys Gly Asp Ile
20 25 30

atc ctt ggg ggg ctc ttt cct att cat ttt gga gta gca gct aaa gat
144
Ile Leu Gly Gly Leu Phe Pro Ile His Phe Gly Val Ala Ala Lys Asp
35 40 45

caa gat ctc aaa tca agg ccg gag tct gtg gaa tgt atc agg tat aat
192
Gln Asp Leu Lys Ser Arg Pro Glu Ser Val Glu Cys Ile Arg Tyr Asn
50 55 60

ttc cgt ggg ttt cgc tgg tta cag gct atg ata ttt gcc ata gag gag
240
Phe Arg Gly Phe Arg Trp Leu Gln Ala Met Ile Phe Ala Ile Glu Glu
65 70 75 80

ata aac agc agc cca gcc ctt ctt ccc aac ttg acg ctg gga tac agg

288
Ile Asn Ser Ser Pro Ala Leu Leu Pro Asn Leu Thr Leu Gly Tyr Arg
85 90 95

ata ttt gac act tgc aac acc gtt tct aag gcc ttg gaa gcc acc ctg
336
Ile Phe Asp Thr Cys Asn Thr Val Ser Lys Ala Leu Glu Ala Thr Leu
100 105 110

agt ttt gtt gct caa aac aaa att gat tct ttg aac ctt gat gag ttc
384
Ser Phe Val Ala Gln Asn Lys Ile Asp Ser Leu Asn Leu Asp Glu Phe
115 120 125

tgc aac tgc tca gag cac att ccc tct acg att gct gtg gtg gga gca
432
Cys Asn Cys Ser Glu His Ile Pro Ser Thr Ile Ala Val Val Gly Ala
130 135 140

act ggc tca ggc gtc tcc acg gca gtg gca aat ctg ctg ggg ctc ttc
480
Thr Gly Ser Gly Val Ser Thr Ala Val Ala Asn Leu Leu Gly Leu Phe
145 150 155 160

tac att ccc cag gtc agt tat gcc tcc tcc agc aga ctc ctc agc aac
528
Tyr Ile Pro Gln Val Ser Tyr Ala Ser Ser Ser Arg Leu Leu Ser Asn
165 170 175

aag aat caa ttc aag tct ttc ctc cga acc atc ccc aat gat gag cac
576
Lys Asn Gln Phe Lys Ser Phe Leu Arg Thr Ile Pro Asn Asp Glu His
180 185 190

cag gcc act gcc atg gca gac atc atc gag tat ttc cgc tgg aac tgg
624
Gln Ala Thr Ala Met Ala Asp Ile Ile Glu Tyr Phe Arg Trp Asn Trp
195 200 205

gtg ggc aca att gca gct gat gac gac tat ggg cgg ccg ggg att gag
672
Val Gly Thr Ile Ala Ala Asp Asp Asp Tyr Gly Arg Pro Gly Ile Glu
210 215 220

aaa ttc cga gag gaa gct gag gaa agg gat atc tgc atc gac ttc agt
720
Lys Phe Arg Glu Glu Ala Glu Glu Arg Asp Ile Cys Ile Asp Phe Ser
225 230 235 240

gaa ctc atc tcc cag tac tct gat gag gaa gag atc cag cat gtg gta
768
Glu Leu Ile Ser Gln Tyr Ser Asp Glu Glu Glu Ile Gln His Val Val
245 250 255

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gag gtg att caa aat tcc acg gcc aaa gtc atc gtg gtt ttc tcc agt
816
Glu Val Ile Gln Asn Ser Thr Ala Lys Val Ile Val Val Phe Ser Ser
            260                 265                 270

ggc cca gat ctt gag ccc ctc atc aag gag att gtc cgg cgc aat atc
864
Gly Pro Asp Leu Glu Pro Leu Ile Lys Glu Ile Val Arg Arg Asn Ile
        275                 280                 285

acg ggc aag atc tgg ctg gcc agc gag gcc tgg gcc agc tcc tcc ctg
912
Thr Gly Lys Ile Trp Leu Ala Ser Glu Ala Trp Ala Ser Ser Ser Leu
    290                 295                 300

atc gcc atg cct cag tac ttc cac gtg gtt ggc ggc acc att gga ttc
960
Ile Ala Met Pro Gln Tyr Phe His Val Val Gly Gly Thr Ile Gly Phe
305                 310                 315                 320

gct ctg aag gct ggg cag atc cca ggc ttc cgg gaa ttc ctg aag aag
1008
Ala Leu Lys Ala Gly Gln Ile Pro Gly Phe Arg Glu Phe Leu Lys Lys
                325                 330                 335

gtc cat ccc agg aag tct gtc cac aat ggt ttt gcc aag gag ttt tgg
1056
Val His Pro Arg Lys Ser Val His Asn Gly Phe Ala Lys Glu Phe Trp
            340                 345                 350

gaa gaa aca ttt aac tgc cac cta cgg cat cta aac ttt aca aac aat
1104
Glu Glu Thr Phe Asn Cys His Leu Arg His Leu Asn Phe Thr Asn Asn
        355                 360                 365

atg ggg gag cag gtg acc ttt gat gag tgt ggt gac ctg gtg ggg aac
1152
Met Gly Glu Gln Val Thr Phe Asp Glu Cys Gly Asp Leu Val Gly Asn
    370                 375                 380

tat tcc atc atc aac tgg cac ctc tcc cca gag gat ggc tcc atc gtg
1200
Tyr Ser Ile Ile Asn Trp His Leu Ser Pro Glu Asp Gly Ser Ile Val
385                 390                 395                 400

ttt aag gaa gtc ggg tat tac aac gtc tat gcc aag aag gga gaa aga
1248
Phe Lys Glu Val Gly Tyr Tyr Asn Val Tyr Ala Lys Lys Gly Glu Arg
                405                 410                 415

ctc ttc atc aac gag gag aaa atc ctg tgg agt ggt ttc tcc agg gag
1296
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Leu Phe Ile Asn Glu Glu Lys Ile Leu Trp Ser Gly Phe Ser Arg Glu
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gtg ccc ttc tcc aac tgc agc cga gac tgc ctg gca ggg acc agg aaa
1344
Val Pro Phe Ser Asn Cys Ser Arg Asp Cys Leu Ala Gly Thr Arg Lys
        435                 440                 445

ggg atc att gag ggg gag ccc acc tgc tgc ttt gag tgt gtg gag tgt
1392
Gly Ile Ile Glu Gly Glu Pro Thr Cys Cys Phe Glu Cys Val Glu Cys
    450                 455                 460

cct gat ggg gag tat agt gat gag aca gat gcc agt gcc tgt aac aag
1440
Pro Asp Gly Glu Tyr Ser Asp Glu Thr Asp Ala Ser Ala Cys Asn Lys
465                 470                 475                 480

tgc cca gat gac ttc tgg tcc aat gag aac cac acc tcc tgc att gcc
1488
Cys Pro Asp Asp Phe Trp Ser Asn Glu Asn His Thr Ser Cys Ile Ala
                485                 490                 495

aag gag atc gag ttt ctg tcg tgg acg gag ccc ttt ggg atc gca ctc
1536
Lys Glu Ile Glu Phe Leu Ser Trp Thr Glu Pro Phe Gly Ile Ala Leu
            500                 505                 510

acc ctc ttt gcc gtg ctg ggc att ttc ctg aca gcc ttt gtg ctg ggt
1584
Thr Leu Phe Ala Val Leu Gly Ile Phe Leu Thr Ala Phe Val Leu Gly
        515                 520                 525

gtg ttt atc aag ttc cgc aac aca ccc att gtc aag gcc acc aac cga
1632
Val Phe Ile Lys Phe Arg Asn Thr Pro Ile Val Lys Ala Thr Asn Arg
    530                 535                 540

gag ctc tcc tac ctc ctc ctc ttc tcc ctg ctc tgc tgc ttc tcc agc
1680
Glu Leu Ser Tyr Leu Leu Leu Phe Ser Leu Leu Cys Cys Phe Ser Ser
545                 550                 555                 560

tcc ctg ttc ttc atc ggg gag ccc cag gac tgg acg tgc cgc ctg cgc
1728
Ser Leu Phe Phe Ile Gly Glu Pro Gln Asp Trp Thr Cys Arg Leu Arg
                565                 570                 575

cag ccg gcc ttt ggc atc agc ttc gtg ctc tgc atc tca tgc atc ctg
1776
Gln Pro Ala Phe Gly Ile Ser Phe Val Leu Cys Ile Ser Cys Ile Leu
            580                 585                 590
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gtg aaa acc aac cgt gtc ctc ctg gtg ttt gag gcc aag atc ccc acc
1824
Val Lys Thr Asn Arg Val Leu Leu Val Phe Glu Ala Lys Ile Pro Thr
        595                 600                 605

agc ttc cac cgc aag tgg tgg ggg ctc aac ctg cag ttc ctg ctg gtt
1872
Ser Phe His Arg Lys Trp Trp Gly Leu Asn Leu Gln Phe Leu Leu Val
    610                 615                 620

ttc ctc tgc acc ttc atg cag att gtc atc tgt gtg atc tgg ctc tac
1920
Phe Leu Cys Thr Phe Met Gln Ile Val Ile Cys Val Ile Trp Leu Tyr
625                 630                 635                 640

acc gcg ccc ccc tca agc tac cgc aac cag gag ctg gag gat gag atc
1968
Thr Ala Pro Pro Ser Ser Tyr Arg Asn Gln Glu Leu Glu Asp Glu Ile
                645                 650                 655

atc ttc atc acg tgc cac gag ggc tcc ctc atg gcc ctg ggc ttc ctg
2016
Ile Phe Ile Thr Cys His Glu Gly Ser Leu Met Ala Leu Gly Phe Leu
            660                 665                 670

atc ggc tac acc tgc ctg ctg gct gcc atc tgc ttc ttc ttt gcc ttc
2064
Ile Gly Tyr Thr Cys Leu Leu Ala Ala Ile Cys Phe Phe Phe Ala Phe
        675                 680                 685

aag tcc cgg aag ctg ccg gag aac ttc aat gaa gcc aag ttc atc acc
2112
Lys Ser Arg Lys Leu Pro Glu Asn Phe Asn Glu Ala Lys Phe Ile Thr
    690                 695                 700

ttc agc atg ctc atc ttc ttc atc gtc tgg atc tcc ttc att cca gcc
2160
Phe Ser Met Leu Ile Phe Phe Ile Val Trp Ile Ser Phe Ile Pro Ala
705                 710                 715                 720

tat gcc agc acc tat ggc aag ttt gtc tct gcc gta gag gtg att gcc
2208
Tyr Ala Ser Thr Tyr Gly Lys Phe Val Ser Ala Val Glu Val Ile Ala
                725                 730                 735

atc ctg gca gcc agc ttt ggc ttg ctg gcg tgc atc ttc ttc aac aag
2256
Ile Leu Ala Ala Ser Phe Gly Leu Leu Ala Cys Ile Phe Phe Asn Lys
            740                 745                 750

atc tac atc att ctc ttc aag cca tcc cgc aac acc atc gag gag gtg
2304
Ile Tyr Ile Ile Leu Phe Lys Pro Ser Arg Asn Thr Ile Glu Glu Val
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        755                 760                 765

cgt tgc agc acc gca gct cac gct ttc aag gtg gct gcc cgg gcc acg
2352
Arg Cys Ser Thr Ala Ala His Ala Phe Lys Val Ala Ala Arg Ala Thr
    770                 775                 780

ctg cgc cgc agc aac gtc tcc cgc aag cgg tcc agc agc ctt gga ggc
2400
Leu Arg Arg Ser Asn Val Ser Arg Lys Arg Ser Ser Ser Leu Gly Gly
785                 790                 795                 800

tcc acg gga tcc acc cct tcc tcc tcc atc agc agc aag agc aac agc
2448
Ser Thr Gly Ser Thr Pro Ser Ser Ser Ile Ser Ser Lys Ser Asn Ser
                805                 810                 815

gaa gac cca ttc cca cag ccc gag agg cag aag cag cag cag ccg ctg
2496
Glu Asp Pro Phe Pro Gln Pro Glu Arg Gln Lys Gln Gln Gln Pro Leu
            820                 825                 830

gcc cta acc cag caa gag cag cag cag cag ccc ctg acc ctc cca cag
2544
Ala Leu Thr Gln Gln Glu Gln Gln Gln Gln Pro Leu Thr Leu Pro Gln
        835                 840                 845

cag caa cga tct cag cag cag ccc aga tgc aag cag aag gtc atc ttt
2592
Gln Gln Arg Ser Gln Gln Gln Pro Arg Cys Lys Gln Lys Val Ile Phe
    850                 855                 860

ggc agc ggc acg gtc acc ttc tca ctg agc ttt gat gag cct cag aag
2640
Gly Ser Gly Thr Val Thr Phe Ser Leu Ser Phe Asp Glu Pro Gln Lys
865                 870                 875                 880

aac gcc atg gcc cac agg aat tct acg cac cag aac tcc ctg gag gcc
2688
Asn Ala Met Ala His Arg Asn Ser Thr His Gln Asn Ser Leu Glu Ala
                885                 890                 895

cag aaa agc agc gat acg ctg acc cga cac cag cca tta ctc ccg ctg
2736
Gln Lys Ser Ser Asp Thr Leu Thr Arg His Gln Pro Leu Leu Pro Leu
            900                 905                 910

cag tgc ggg gaa acg gac tta gat ctg acc gtc cag gaa aca ggt ctg
2784
Gln Cys Gly Glu Thr Asp Leu Asp Leu Thr Val Gln Glu Thr Gly Leu
        915                 920                 925

caa gga cct gtg ggt gga gac cag cgg cca gag gtg gag gac cct gaa
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2832
Gln Gly Pro Val Gly Gly Asp Gln Arg Pro Glu Val Glu Asp Pro Glu
930 935 940

gag ttg tcc cca gca ctt gta gtg tcc agt tca cag agc ttt gtc atc
2880
Glu Leu Ser Pro Ala Leu Val Val Ser Ser Ser Gln Ser Phe Val Ile
945 950 955 960

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965 970

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35 40 45

Gln Asp Leu Lys Ser Arg Pro Glu Ser Val Glu Cys Ile Arg Tyr Asn
50 55 60

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65 70 75 80

Ile Asn Ser Ser Pro Ala Leu Leu Pro Asn Leu Thr Leu Gly Tyr Arg
85 90 95

Ile Phe Asp Thr Cys Asn Thr Val Ser Lys Ala Leu Glu Ala Thr Leu
100 105 110

Ser Phe Val Ala Gln Asn Lys Ile Asp Ser Leu Asn Leu Asp Glu Phe
115 120 125

Cys Asn Cys Ser Glu His Ile Pro Ser Thr Ile Ala Val Val Gly Ala
130 135 140

Thr Gly Ser Gly Val Ser Thr Ala Val Ala Asn Leu Leu Gly Leu Phe
145 150 155 160

Tyr Ile Pro Gln Val Ser Tyr Ala Ser Ser Ser Arg Leu Leu Ser Asn
165 170 175

Lys Asn Gln Phe Lys Ser Phe Leu Arg Thr Ile Pro Asn Asp Glu His
180 185 190

Gln Ala Thr Ala Met Ala Asp Ile Ile Glu Tyr Phe Arg Trp Asn Trp
195 200 205

Val Gly Thr Ile Ala Ala Asp Asp Asp Tyr Gly Arg Pro Gly Ile Glu
210 215 220

Lys Phe Arg Glu Glu Ala Glu Glu Arg Asp Ile Cys Ile Asp Phe Ser
225 230 235 240

Glu Leu Ile Ser Gln Tyr Ser Asp Glu Glu Glu Ile Gln His Val Val
245 250 255

Glu Val Ile Gln Asn Ser Thr Ala Lys Val Ile Val Val Phe Ser Ser
260 265 270

Gly Pro Asp Leu Glu Pro Leu Ile Lys Glu Ile Val Arg Arg Asn Ile
275 280 285

Thr Gly Lys Ile Trp Leu Ala Ser Glu Ala Trp Ala Ser Ser Ser Leu
290 295 300

Ile Ala Met Pro Gln Tyr Phe His Val Val Gly Gly Thr Ile Gly Phe
305 310 315 320

Ala Leu Lys Ala Gly Gln Ile Pro Gly Phe Arg Glu Phe Leu Lys Lys
325 330 335

Val His Pro Arg Lys Ser Val His Asn Gly Phe Ala Lys Glu Phe Trp
340 345 350

Glu Glu Thr Phe Asn Cys His Leu Arg His Leu Asn Phe Thr Asn Asn
355 360 365

Met Gly Glu Gln Val Thr Phe Asp Glu Cys Gly Asp Leu Val Gly Asn
370 375 380

Tyr Ser Ile Ile Asn Trp His Leu Ser Pro Glu Asp Gly Ser Ile Val
385 390 395 400

Phe Lys Glu Val Gly Tyr Tyr Asn Val Tyr Ala Lys Lys Gly Glu Arg
405 410 415

Leu Phe Ile Asn Glu Glu Lys Ile Leu Trp Ser Gly Phe Ser Arg Glu
420 425 430

Val Pro Phe Ser Asn Cys Ser Arg Asp Cys Leu Ala Gly Thr Arg Lys
435 440 445

Gly Ile Ile Glu Gly Glu Pro Thr Cys Cys Phe Glu Cys Val Glu Cys

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    450                 455                 460

Pro Asp Gly Glu Tyr Ser Asp Glu Thr Asp Ala Ser Ala Cys Asn Lys
465                 470                 475                 480

Cys Pro Asp Asp Phe Trp Ser Asn Glu Asn His Thr Ser Cys Ile Ala
                485                 490                 495

Lys Glu Ile Glu Phe Leu Ser Trp Thr Glu Pro Phe Gly Ile Ala Leu
            500                 505                 510

Thr Leu Phe Ala Val Leu Gly Ile Phe Leu Thr Ala Phe Val Leu Gly
        515                 520                 525

Val Phe Ile Lys Phe Arg Asn Thr Pro Ile Val Lys Ala Thr Asn Arg
    530                 535                 540

Glu Leu Ser Tyr Leu Leu Leu Phe Ser Leu Leu Cys Cys Phe Ser Ser
545                 550                 555                 560

Ser Leu Phe Phe Ile Gly Glu Pro Gln Asp Trp Thr Cys Arg Leu Arg
                565                 570                 575

Gln Pro Ala Phe Gly Ile Ser Phe Val Leu Cys Ile Ser Cys Ile Leu
            580                 585                 590

Val Lys Thr Asn Arg Val Leu Leu Val Phe Glu Ala Lys Ile Pro Thr
        595                 600                 605

Ser Phe His Arg Lys Trp Trp Gly Leu Asn Leu Gln Phe Leu Leu Val
    610                 615                 620

Phe Leu Cys Thr Phe Met Gln Ile Val Ile Cys Val Ile Trp Leu Tyr
625                 630                 635                 640

Thr Ala Pro Pro Ser Ser Tyr Arg Asn Gln Glu Leu Glu Asp Glu Ile
                645                 650                 655

Ile Phe Ile Thr Cys His Glu Gly Ser Leu Met Ala Leu Gly Phe Leu
            660                 665                 670

Ile Gly Tyr Thr Cys Leu Leu Ala Ala Ile Cys Phe Phe Phe Ala Phe
        675                 680                 685

Lys Ser Arg Lys Leu Pro Glu Asn Phe Asn Glu Ala Lys Phe Ile Thr
    690                 695                 700

Phe Ser Met Leu Ile Phe Phe Ile Val Trp Ile Ser Phe Ile Pro Ala
705                 710                 715                 720

Tyr Ala Ser Thr Tyr Gly Lys Phe Val Ser Ala Val Glu Val Ile Ala
                725                 730                 735
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Ile Leu Ala Ala Ser Phe Gly Leu Leu Ala Cys Ile Phe Phe Asn Lys
740 745 750

Ile Tyr Ile Ile Leu Phe Lys Pro Ser Arg Asn Thr Ile Glu Glu Val
755 760 765

Arg Cys Ser Thr Ala Ala His Ala Phe Lys Val Ala Ala Arg Ala Thr
770 775 780

Leu Arg Arg Ser Asn Val Ser Arg Lys Arg Ser Ser Ser Leu Gly Gly
785 790 795 800

Ser Thr Gly Ser Thr Pro Ser Ser Ser Ile Ser Ser Lys Ser Asn Ser
805 810 815

Glu Asp Pro Phe Pro Gln Pro Glu Arg Gln Lys Gln Gln Gln Pro Leu
820 825 830

Ala Leu Thr Gln Gln Glu Gln Gln Gln Gln Pro Leu Thr Leu Pro Gln
835 840 845

Gln Gln Arg Ser Gln Gln Gln Pro Arg Cys Lys Gln Lys Val Ile Phe
850 855 860

Gly Ser Gly Thr Val Thr Phe Ser Leu Ser Phe Asp Glu Pro Gln Lys
865 870 875 880

Asn Ala Met Ala His Arg Asn Ser Thr His Gln Asn Ser Leu Glu Ala
885 890 895

Gln Lys Ser Ser Asp Thr Leu Thr Arg His Gln Pro Leu Leu Pro Leu
900 905 910

Gln Cys Gly Glu Thr Asp Leu Asp Leu Thr Val Gln Glu Thr Gly Leu
915 920 925

Gln Gly Pro Val Gly Gly Asp Gln Arg Pro Glu Val Glu Asp Pro Glu
930 935 940

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acc tct gcc tac ggg cca gac cag cga gcc caa aag aag ggg gac att   96

Thr Ser Ala Tyr Gly Pro Asp Gln Arg Ala Gln Lys Lys Gly Asp Ile
             20                  25                  30

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144
Ile Leu Gly Gly Leu Phe Pro Ile His Phe Gly Val Ala Ala Lys Asp
         35                  40                  45

caa gat ctc aaa tca agg ccg gag tct gtg gaa tgt atc agg tat aat
192
Gln Asp Leu Lys Ser Arg Pro Glu Ser Val Glu Cys Ile Arg Tyr Asn
     50                  55                  60

ttc cgt ggg ttt cgc tgg tta cag gct atg ata ttt gcc ata gag gag
240
Phe Arg Gly Phe Arg Trp Leu Gln Ala Met Ile Phe Ala Ile Glu Glu
 65                  70                  75                  80

ata aac agc agc cca gcc ctt ctt ccc aac ttg acg ctg gga tac agg
288
Ile Asn Ser Ser Pro Ala Leu Leu Pro Asn Leu Thr Leu Gly Tyr Arg
                 85                  90                  95

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Ile Phe Asp Thr Cys Asn Thr Val Ser Lys Ala Leu Glu Ala Thr Leu
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agt ttt gtt gct caa aac aaa att gat tct ttg aac ctt gat gag ttc
384
Ser Phe Val Ala Gln Asn Lys Ile Asp Ser Leu Asn Leu Asp Glu Phe
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Cys Asn Cys Ser Glu His Ile Pro Ser Thr Ile Ala Val Val Gly Ala
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Gln Ala Thr Ala Met Ala Asp Ile Ile Glu Tyr Phe Arg Trp Asn Trp
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2352
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Ile Asn Ser Ser Pro Ala Leu Leu Pro Asn Leu Thr Leu Gly Tyr Arg
                 85                  90                  95

Ile Phe Asp Thr Cys Asn Thr Val Ser Lys Ala Leu Glu Ala Thr Leu
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17116

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/705, 16/28; C12N 5/10, 15/12

US CL :435/7.1, 69.1, 252.3, 320.1; 350/350, 388.22; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 69.1, 252.3, 320.1; 350/350, 388.22; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN/MEDLINE
search terms: calcium, sensing, receptor#, exon#

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A --- X	FREICHEL et al. Expression of a Calcium-Sensing Receptor in a Human Medullary Thyroid Carcinoma Line and Its Contribution to Calcitonin Secretion. Endocrinology. September 1996, Vol. 137, No. 9, pages 3842-3848, see entire document.	1-29, 34-50 ---------- 30-33
A, P --- X, P	ODA et al. The Calcium Sensing Receptor and Its Alternatively Spliced Form in Keratinocyte Differentiation. The Journal of Biological Chemistry. 04 September 1998, Vol. 273, No. 36, pages 23344-23352, see entire document.	35-37 ---------- 1-34, 38-50

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 SEPTEMBER 1999	Date f mailing of the international search report 28 OCT 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JOHN D. ULM Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

PCT

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(51) International Patent Classification 6 :
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A1

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60/094,702 30 July 1998 (30.07.98) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/094,702 (CIP)
Filed on 30 July 1998 (30.07.98)

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(72) Inventors; and
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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: ISOFORMS OF HUMAN CALCIUM SENSING RECEPTOR

(57) Abstract

The present invention relates to isoforms of a human calcium sensing receptor, and to the genes encoding these isoforms. The invention further relates to methods of screening for agonists or antagonists of the isoforms, particularly with respect to calcium receptor activity, to diagnostic uses of these isoforms and to therapeutic uses of the agonists or antagonists. The invention also relates to gene therapy using the genes encoding the receptor isoforms or molecules capable of down–regulating receptor activity, such as antisense sequences.